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Perspectives on Pathogenic Neisseriae

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The Sixth International Pathogenic Neisseriae Conference was held at Callaway Gardens Conference Center, Pine Mountain, GA., 16 to 21 October 1988. The primary goal of the organizers of the conference was to bring together investigators, postdoctoral fellows and graduate students from academia, government, and industry who are involved with or interested in Neisseria gonorrhoeae and Neisseria meningitidis to shart ongoing reserach on these organisms and the diseases they cause, to communicate with fellow investigators, and to stimulate potential collaborative endeavors. (rn) ←

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Preface

The Sixth International Pathogenic Neisseriae Conference was held at Callaway Gardens Conference Center, Pine Mountain, Ga., 16 to 21 October 1988. This conference followed similar meetings in San Francisco, Calif. (1978); Hemavan, Sweden (1980); Montreal, Canada (1982); Asilomar, Calif. (1984); and Noordwijkerhout, The Netherlands (1986). The primary goal of the organizers of the conference was to bring together investigators, postdoctoral fellows and graduate students from academia, government, and industry who are involved with or interested in *Neisseria gonorrhoeae* and *Neisseria meningitidis* to share ongoing research on these organisms and the diseases they cause, to communicate with fellow investigators, and to stimulate potential collaborative endeavors. The success of previous meetings in these regards is evidenced throughout the following review articles. Scientists from 24 countries attended this conference. There were 47 oral presentations and 140 posters covering topics such as pathogenesis, molecular biology, epidemiology, immunity, diagnostic tests, and vaccine development.

Past conference organizers have published the oral and poster presentations of each meeting. While these publications were generally useful, they had several disadvantages. First, publication of research articles in symposium volumes often precluded publication of the same data in a primary journal. Second, many of the papers were not reviewed by the standards applied to manuscripts submitted to journals. Third, inherent and sometimes unforeseen difficulties in meeting publication schedules often resulted in unfortunate delays in getting the articles printed. After consultation with the American Society for Microbiology, the current organizers decided to solicit review articles from leaders in the field on topics that would broadly cover the major areas of gonococcal and meningococcal research. These articles summarize the major advances in each field and indicate those areas that require further study.

Gonococcal and meningococcal research has begun a new era. Investigators have recently agreed on a uniform nomenclature system for cellular components that it is hoped will avoid some of the confusion in the literature. Advances in the molecular biology and genetics of *N. gonorrhoeae* and *N. meningitidis* have necessitated the article on genetic loci and linkage associations. It will probably not be too long before we have an adequate genetic map for these organisms. There has been remarkable progress in our understanding of antigenic variation in *N. gonorrhoeae*. These advances have heightened the difficulties that have been, and will be, encountered in developing an effective vaccine against gonorrhea. It is important also to note that studies on the pathogenic *Neisseria* spp. have led to fundamental concepts about mucosal pathogens in general and have stimulated research on other bacterial pathogens.

The Organizing Committee is grateful to the following organizations and companies for their support of this conference: Centers for Disease Control; Emory University; National Institute of Allergy and Infectious Diseases; Food and Drug Administration; U.S. Army; Miles, Inc./Bayer AG; SYVA Co.; Institut Merieux; Upjohn Pharmaceuticals; Hoffmann-La Roche Inc.; Tambrands/ Hygeia Sciences; Merck Sharp & Dohme; Hoechst-Roussel Pharmaceuticals Inc.; Marion Laboratories, Inc.; Roerig Division of Pfizer, Inc.; Lederle Laboratories; and Eastman Kodak Co. The Organizing Committee also wishes to express its appreciation to the Publications Board of the American Society for Microbiology and to the editor of *Clinical Microbiology Reviews* for making this publication possible.

The Organizing Committee

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Conserved Lipoproteins of Pathogenic *Neisseria* Species Bearing the H.8 Epitope: Lipid-Modified Azurin and H.8 Outer Membrane Protein

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Many of the surface components of the pathogenic *Neisseria* species demonstrate marked antigenic variability, both between different strains and among variants of a single strain. Numerous examples of such variable surface components are described in the other reviews in this issue. Amid this surface variability, there are relatively few components or antigens that are conserved among all gonococci or meningococci, or both. The conserved antigens that have been identified have been the subject of considerable study in an effort to determine whether they play a direct role in neisserial pathogenesis and whether they might be targets of a protective immune response. The existence of a protein epitope that is conserved among pathogenic *Neisseria* species was revealed by the binding of a monoclonal antibody (MAb) designated H.8 (8), as well as other MAbs with similar specificities (14, 31). These MAbs bind to all gonococci and meningococci that have been tested and to *N. lactamica* and *N. cinerea* strains, but not to strains of other commensal *Neisseria* species (1, 8, 14, 31). There has been much recent progress in identifying and characterizing the proteins that are recognized by H.8-specific MAbs, particularly through the use of recombinant deoxyribonucleic acid (DNA) approaches. The existence of multiple proteins that bind H.8-specific MAbs was revealed by screening libraries of gonococcal and meningococcal genes. Gotschlich et al. (11) cloned two distinct gonococcal genes that hybridize to each other in Southern blotting experiments and encode H.8 MAb-binding proteins with similar apparent molecular weights. Further analysis of these and other cloned genes has added to our understanding of the characteristics of two different proteins that are recognized by H.8-specific MAbs: the lipid-modified azurin and the H.8 outer membrane protein (designated Laz and Lip, respectively, in the nomenclature recommendations included in this issue).

LIPID-MODIFIED AZURIN (Laz)

The Laz protein is a two-domain lipoprotein that is highly conserved in gonococci and meningococci. The gene encoding Laz has been cloned and sequenced from both a gonococcal (11, 12) and a meningococcal (17) strain; the two genes are virtually identical. The DNA sequence of the *laz* gene predicts that the protein has the following features. (i) The first is a most probable signal peptide-processing site matching the consensus site for bacterial lipoproteins. In other procaryotic lipoproteins, this site is recognized and processed by signal peptidase II, resulting in an N-terminal cysteine residue modified with glycerol and fatty acid (21, 30). The activity of signal peptidase II is specifically inhibited by the cyclic peptide antibiotic globomycin (15). Using the cloned meningococcal *laz* gene in *Escherichia coli*, we showed that the Laz protein could be modified with radiolabeled palmitic acid and that its processing was sensitive to inhibition by globomycin, thus confirming the similarity of the recombinant-derived neisserial protein to other procaryotic lipoproteins (J. P. Woods, J. F. Dempsey, T. H. Kawula, D. S. Barritt, and J. G. Cannon, Mol. Microbiol., in press).

(ii) The predicted mature Laz protein has a molecular mass of 17 kilodaltons. The N-terminal 39 amino acids of the mature protein form a domain composed primarily of imperfect repeats of the sequence alanine-alanine-glutamate-alanine-proline (AAEAP). The epitope for H.8 MAb binding was localized to no more than 20 amino acids within this region by analysis of deletion subclones of the meningococcal *laz* gene and by analysis of MAb binding to synthetic peptides (17). Thus, the epitope for binding of the H.8 MAb is linear and is not dependent on the covalent lipid modification of the protein. There is little or no visible Laz signal when Western immunoblots of gonococci or meningococci are probed with the H.8 MAb, suggesting that the binding of the MAb to Laz is below the threshold of detection by this technique (11; Woods et al., in press). The presence of the H.8 epitope on Laz was revealed only when the genes were cloned on plasmid (17) or bacteriophage (11) vectors, thus amplifying signal intensity.

(iii) The remainder of the Laz protein (127 amino acids) has striking similarity to the sequence of azurins, which are small, blue, copper-containing proteins that are believed to function in electron transport during respiration (10, 16, 25, 26). Azurins from *Pseudomonas*, *Alcaligenes*, and *Bordetella* species show considerable amino acid sequence homology, including 48 of 129 amino acids that are completely conserved among nine sequenced azurins (26). When the 127 C-terminal acids of Laz are compared with a composite azurin sequence, 77% of the amino acids are homologous. Of the 48 conserved azurin amino acids, 42 (88%) are identical in the predicted Laz protein, including the 4 azurin amino acids forming the copper-binding site (22). The azurins from other bacterial genera are not lipoproteins and do not have the 39-amino-acid N-terminal domain that is present in the neisserial Laz protein (9, 25, 26).

In addition to DNA sequence similarity, there is other evidence confirming the similarity of the neisserial Laz protein to azurins. Gonococcal Laz has been purified from an *E. coli* lysogen containing the cloned *laz* gene, and the spectral characteristics of the resulting blue protein are the same as those reported for conventional azurins (E. Gotschlich, personal communication). Also, the gonococcal and meningococcal Laz proteins cross-react with antiserum to purified *Pseudomonas* azurin. Commensal *Neisseria* species also produce an azurinlike protein recognized by the anti-azurin serum. The characteristics of the azurins from commensal species have not been completely determined, but the proteins may differ in some features from the Laz of the pathogens (Woods et al., in press).

What is the function of the Laz protein? In other bacterial genera, azurins function in electron transfer, most probably between cytochrome c_{551} and cytochrome oxidase (10, 25). Gotschlich and Seiff (12) suggested that the azurinlike domain of Laz might function in electron transport during anaerobic growth of gonococci or meningococci, which can occur if nitrite is provided as a terminal electron acceptor (18). If the Laz protein does play such a role, a mutant lacking Laz might be expected to be unable to grow under anaerobic conditions. Gotschlich has constructed such a mutant of gonococcal strain F62 by insertionally inactivating the cloned *laz* gene and introducing the mutated gene by transformation into the gonococcus. The Laz$^-$ mutants grow under both aerobic and anaerobic conditions in the presence of nitrite (Gotschlich, personal communication). It is still possible that Laz functions in electron transport via a pathway that has not yet been identified in gonococci or meningococci. Since both pathogenic and commensal *Neisseria* species produce Laz or a protein similar to it, it seems unlikely that Laz will have a specific role in enhancing the virulence of gonococci and meningococci.

H.8 OUTER MEMBRANE PROTEIN (Lip)

When gonococcal or meningococcal outer membrane preparations are subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with H.8-specific MAbs in a Western blot, a MAb-binding band with unusual electrophoretic characteristics is observed. On one-dimensional gels, the antigen forms a cone-shaped band with an apparent molecular mass ranging from 18 to 30 kilodaltons (8, 14, 17). On two-dimensional gels, the antigen migrates away from diagonal (14). The protein responsible for this MAb-binding signal, designated the H.8 outer membrane protein, H.8 antigen, or Lip (for lipoprotein), does not stain with Coomassie blue and stains with silver only under some conditions (2, 8, 14). The apparent molecular mass of Lip varies in different strains, but appears to be constant within a single gonococcal or meningococcal strain (8, 14).

Two different methods for the purification of Lip have been published. Strittmatter and Hitchcock (28) used phenol-chloroform-petroleum ether extraction of gonococci, followed by several chromatographic steps, to obtain purified Lip. The protein copurifies with two lipid components, is alanine and proline rich, and lacks aromatic amino acids and methionine. Bhattacharjee et al. (2) purified meningococcal Lip by Empigen BB extraction and high-performance liquid chromatography. The purified protein is rich in glutamate, alanine, and proline and contains a lipid component that elutes between C_9 and C_{11} straight-chain fatty acid standards in the gas chromatograph. The unusual amino acid composition of the protein is consistent with its lack of absorbance at 280 nm (2, 28). The pattern of biosynthetic labeling of Lip with radiolabeled amino acids and fatty acids is consistent with the composition determined for purified Lip protein (W. Baehr, E. C. Gotschlich, and P. J. Hitchcock, Mol. Microbiol., in press).

Recent studies on the cloning and sequencing of gonococcal *lip* genes have clarified the structure of this unusual protein. Gotschlich et al. (11) produced two different λgt11 clones containing the *lip* gene of gonococcal strain R10, and those clones have been sequenced (Baehr et al., in press). The *lip* gene from strain FA1090 was cloned in a lambda bacteriophage vector (5) and sequenced by Woods et al. (J. P. Woods, S. M. Spinola, S. M. Strobel, and J. G. Cannon, Mol. Microbiol., in press). The DNA sequence of the *lip* gene in the two strains is quite similar. In each case, the DNA sequence predicts that Lip is a lipoprotein, with a lipoprotein signal peptide-processing site like that of the Laz protein. The N-terminal amino acid of mature Lip protein in both strains is a cysteine residue. In FA1090, the predicted protein is 71 amino acids in length, composed entirely of 13 repeats of the AAEAP consensus sequence that was also found in the epitope-encoding region of Laz (12, 17). Perfect five-residue periodicity is maintained throughout the protein. Seven of the repeats match the consensus sequence exactly, five have single amino acid substitutions, and one has two amino acid substitutions. Only six of the amino acids in the mature protein are not included in the repetitive sequence. In strain R10, the predicted protein is 76 amino acids long, consisting of 14 AAEAP repeats. Ten of the repeats are perfect. It is possible that differences in the number of AAEAP repeats contribute to the differences in apparent molecular mass of Lip in different strains. Despite the high proline content of the protein, the predicted secondary structure is α-helical. The predicted molecular mass of the polypeptide portion of FA1090 Lip is 6.3 kilodaltons but the protein migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an apparent molecular mass of 20 kilodaltons. The aberrant migration of the protein may be due to its lipid modification or to its unusual amino acid composition and repeating structure. The sequence of a meningococcal *lip* gene has not yet been determined.

Although Lip is noteworthy in the perfect conservation of most of the AAEAP repeats, there are other microbial proteins with similar repetitive motifs. Streptococcal M protein contains imperfect heptapeptide repeats making up part of the protein. The repetitive nature of this region is important in formation of an α-helical coiled-coil structure and in the antiphagocytic function of the protein (19, 23). Another protein with a repetitive structure is the major lipoprotein (Lpp) of *E. coli*, which has several intriguing similarities to gonococcal Lip. In both proteins, the polypeptide is relatively small (58 amino acids for Lpp) and has an N-terminal cysteine and a C-terminal lysine residue (21, 30). The linkage of Lpp to peptidoglycan is through the ε-amino group of the C-terminal lysine (7). In Lpp, a seven-amino-acid periodicity is important in the formation of the characteristic coiled-coil structure of the protein (6, 20). The seven-amino-acid repeats in the *E. coli* Lpp are not as highly conserved as the AAEAP repeats in Lip. It is not known whether Lip serves a structural role in the outer membrane similar to that of Lpp or whether it is peptidoglycan associated under some or all growth conditions.

ROLE OF H.8 (Lip) IN NEISSERIAL PATHOGENESIS AND IMMUNITY

The association of the H.8 epitope with the pathogenic *Neisseria* species has led to speculation that the antigens bearing it might be involved in neisserial pathogenesis or that they might be effective as components of a vaccine for prevention of gonococcal or meningococcal disease. The majority of H.8 MAb binding is to the Lip protein, which consists essentially of a repeating H.8 epitope. There are conflicting data about whether the epitope recognized by the MAb is exposed on the surface of intact, viable organisms. For gonococci, experiments involving adsorption of MAb from solution (8), fluorescent-antibody labeling (14), slide agglutination (14), and immunoelectron microscopy (14) have been interpreted as evidence for surface exposure. However, other studies involving immunoelectron micros-

copy (24) showed that the majority of organisms do not mark with gold-conjugated MAb, although outer membrane blebs label heavily. In any of these studies, the apparent surface exposure of the target epitope could be influenced by the conditions used for preparation and fixation of the cells, and it is difficult to draw firm conclusions about this issue. Lip is immunogenic in patients with localized or disseminated gonococcal infections (3, 4, 13) and in patients with meningococcal disease (3). It is not known whether an antibody response directed at Lip would protect against infection. In the mouse model of meningococcal infection, MAb specific for Lip was not protective (29). However, the MAb used in those experiments was not bactericidal, leaving the possibility open that a cidal Lip-specific MAb would be protective. Also, Schweinle et al. have obtained data indicating that MAb 10, which is specific for the H.8 epitope, is bactericidal and opsonic for some gonococcal strains (J. E. Schweinle, P. J. Hitchcock, A. J. Tenner, C. H. Hammer, M. M. Frank, and K. A. Joiner, J. Clin. Invest., in press).

The recent construction of a gonococcal mutant lacking the Lip protein should allow better determination of the role of this protein in neisserial physiology and virulence (Woods et al., in press). In this mutant, the gene encoding Lip was insertionally inactivated by using the shuttle mutagenesis system developed for gonococci by Seifert et al. (27). The Lip-less mutant shows no alteration in its growth rate in rich medium and is unaltered in its serum resistance.

It may be necessary to reevaluate the available data about the association of Lip with pathogenic *Neisseria* species in light of recent information gained about the structure of the protein. When commensal *Neisseria* species are probed with H.8-specific MAbs or with DNA probes derived from the *lip* gene, they are negative for MAb binding or DNA hybridization (1, 5, 8). However, since Lip consists only of repeats of a single unit, encoded by a similarly repeating DNA sequence, it is possible that commensal *Neisseria* species have a different protein with a slightly different repeat unit. Such a protein would not be detected by the MAb and DNA probes derived from *lip* of the pathogens. The association of the H.8 epitope with pathogenic *Neisseria* species may be a reflection of clonal relationships among *Neisseria* species, rather than a consequence of a direct role of the protein in pathogenesis. The question of the role of Lip in gonococcal or meningococcal pathogenesis is still open, and further experiments are needed to clarify the function of this unusual lipoprotein in pathogenic *Neisseria* species.

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Humoral Immune Response to Gonococcal Infections

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The purpose of this paper is to review the recent advances in the knowledge of humoral immune responses to gonococcal infections in humans. The current knowledge of the molecular biology of *Neisseria gonorrhoeae*, including knowledge of antigen structure and mechanisms that yield antigenic heterogeneity, has outstripped the current knowledge of immune responses to the gonococcal antigens. Although it is clear that patients with uncomplicated infections develop increased levels of serum antigonococcal immunoglobulins, it is also evident that because of the new knowledge about antigenic heterogeneity, generalized interpretation of results from early studies often is not appropriate. Antibodies that are reactive with a given gonococcal antigen may not react at all with the same antigen from another strain of *N. gonorrhoeae*. Thus, in this review we emphasize data that have taken into account the antigenic heterogeneity or have included conserved antigens of the gonococcus.

SERUM ANTIBODY RESPONSE IN GONOCOCCAL INFECTION

Immunoglobulin Class

In the late 1960s, several studies of natural serum antibodies reactive with *N. gonorrhoeae* and other gram-negative organisms were reported (14–17). Indirect fluorescent-antibody assays were used and showed reactive immunoglobulin G (IgG) in adult sera and in umbilical-cord sera. Less IgM that was reactive and relatively little reactive IgA were found in the sera from adults. The IgG in immune sera could be distinguished from naturally occurring IgG antibodies by reaction with heat-labile gonococcal antigens (14). Similarly, 9 of 10 men with experimental gonococcal urethritis developed significant increases in reactive IgG levels in serum (15); fewer of the patients showed increased levels of reactive IgM or IgA. Serum IgA reactive with *N. gonorrhoeae* probably is secretory, implying that mucosal cells are the origin of the antibody (22).

IgG3 is the predominant IgG subclass reactive with a variety of gonococcal antigens, followed by IgG1 and IgG4 (34). There is minimal IgG2 reactive with gonococcal antigens following infection, suggesting that polysaccharides are not important in the immune response to gonorrhea.

Antigen Specificity

Antigenic heterogeneity is a major consideration when studying humoral immunity in gonococcal infection. Pili, protein II (PII), and lipooligosaccharide (LOS) are the most important antigens, quantitatively, in generating antibody responses in gonococcal infection. These three antigens shift from one antigenic form to another at a frequency of 1 in 10^3, or greater. Because of the rapid shifting from one antigenic form to another, nearly every strain of *N. gonorrhoeae* may be antigenically distinct. Indeed, it is possible that each gonococcus has the potential to be antigenically distinct from its neighbors. The molecular biology of pili (25a), protein I (PI) (28a), PII (42a), protein III (PIII) (7a), and H.8 (12a) is discussed elsewhere in this issue. Humoral immunity related to these antigens is discussed below.

Pili. Pili are the hairlike appendages that extend from the gonococcal cell surface and are thought to function in the attachment of gonococci to host cells. Soon after the description of gonococcal pili in 1971, it was noted that patients make antibodies against pili, as measured by using pili from a laboratory strain of *N. gonorrhoeae* as the test antigen (12). The observations were repeated by several investigators. One study noted that there were differences in the antibody levels between men and women, although there was some overlap; the antibody levels were related to the number of previous gonococcal infections, but, again, there was a great deal of overlap; and black Americans tended to have different levels from those of white Americans (20). Thus, gonococcal pili have not proven to be a useful reagent in development of a serological test to diagnose gonorrhea.

It is clear that patients make antibodies against the pili of the infecting gonococcal strain (36). In women, pili appeared to be the predominant antigen in the immune response. In men, there were higher levels of antibodies to other antigens than pili.

One consideration of humoral immunity to pili was that antibodies against pili blocked the pilus-mediated attachment to host cells (8, 59). A vaccine was developed by using pili from a laboratory strain of *N. gonorrhoeae* (8, 59), but a field trial of the vaccine showed it not to be efficacious (J. Boslego, R. Chung, J. Sadoff, D. McChesney, M. Piziak, J. Ciak, J. Brown, W. Caldwell, D. Berliner, G. Seitter, C. Brinton, and E. Tramont, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 294, 1984). Thus, although there is a strong humoral immune response to gonococcal pili, the antigenic heterogeneity of the pilin molecule makes development of a useful pilus vaccine difficult. Antibodies against synthetic peptides representing conserved regions of the pilin molecule appear to have biological activity in blocking the pilus-mediated attachment of gonococci to host cells (48); however, persons immunized with a gonococcal pilus vaccine lack antibodies against such synthetic peptides (R. Chung, C. Liu, J. Boslego, E. Tramont, S. Wood, and C. Brinton, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B5, p. 25). To date, there are no data on the human immune response to immunization with synthetic peptides representing gonococcal antigens.

PI. PI is a major component of the gonococcal outer membrane and may have an important role in the pathogenesis of infection by insertion into the host cell membrane (7, 13). PI is antigenically conserved in each strain of *N. gonorrhoeae* but variable from one strain to another. There are two primary types, PIA and PIB, each having multiple serotypes (32, 33, 49). PIA strains are usually resistant to the bactericidal action of normal human serum, whereas PIB strains often are susceptible (29, 32).

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Patients with uncomplicated gonococcal genital infection, pelvic inflammatory disease, or disseminated infection have detectable serum antibodies reactive with PI (27, 28, 36). The anti-PI antibodies may be bactericidal for the gonococci (27). The antibody response to PI, however, is minimal when compared with the response to pili, PII, and LOS, especially given the large amount of PI in the gonococcal outer membrane.

PII. PIIs are variably expressed surface-exposed gonococcal proteins (3, 18, 56). At any one time a gonococcal strain may have no, one, two, or many PIIs in the outer membrane.

PII is one of the most important antigens in the humoral immune response to gonococcal infection in both men and women (36). Men and women infected with the same strain of *N. gonorrhoeae*, however, may have different immune responses to a specific PII or may develop antibodies to different PIIs (35). A biological role for anti-PII antibodies in gonococcal infection is undefined. One possible role is that by shifting expression of PII, gonococci may evade the immune system of the host.

PIII. PIII is a protein that is antigenically conserved in all gonococci. PIII is closely associated with PI in the outer membrane, but a specific function for PIII is not known.

Patients with gonococcal infection make small amounts of antibody to PIII (36). Whether there is any biological role for these antibodies in protection from infection is unknown. It has been demonstrated, however, that IgG reactive with PIII blocks the serum bactericidal action in disseminated gonococcal infection (47).

LOS. The LOS of gonococci is a relatively small form of bacterial endotoxin with molecular weights of 3,200 to 7,100 (24, 51). Each gonococcal strain can express several different antigenic forms of LOS at one time and can very rapidly switch from one antigenic form to another (38, 52). The LOS appears to be tightly associated with outer membrane proteins I and II (7, 26). Gonococcal LOS also has structures that are immunochemically similar to structures on human erythrocyte membranes (39). Because of its antigenic diversity and structure, association with outer membrane proteins, and endotoxic activity, gonococcal LOS has an important role in the immunology of gonorrhea.

Genital infection with *N. gonorrhoeae* elicits serum anti-LOS antibody directed against the LOS of the infecting strain (28, 36). Not all patients have serum antibody directed against the LOS present after subculture of the infecting strain, perhaps because of shifting of the antigenic forms of LOS on subculture. Patients do have more antibody against the LOS of their infecting strains than against the LOS of laboratory strains (1). Disseminated gonococcal infection elicits higher levels of anti-LOS antibody than genital infection does (37).

Antibody against LOS has several important functions in gonococcal infection. Antibody can activate complement through the classical or alternative complement pathways and can be chemotaxic for polymorphonuclear cells (19, 25). IgM and IgG directed against LOS can be bactericidal for gonococci; IgA can block the IgG-mediated bactericidal activity (2). Gonococci can express at least one antigenic form that confers resistance to the bactericidal action of normal human serum (50, 54); antibody to this form of LOS can be bactericidal, but that antibody is seldom present in human serum.

H.8. H.8 is a distinctive antigen common to the pathogenic neisseriae (23, 57). Patients with uncomplicated genital gonococcal infection, pelvic inflammatory disease, or disseminated infection make antibodies against H.8 (4, 5, 36). It is also evident that antibodies against H.8 do not protect the host from uncomplicated genital infections, since patients can have repeated infection when anti-H.8 antibodies are present.

IgA1 protease. The pathogenic *Neisseria* spp. produce extracellular IgA1 protease, which cleaves IgA1 and inactivates it (55). In preliminary observations it was found that only 8 of 48 patients with uncomplicated gonococcal infection, gonococcal pelvic inflammatory disease, or disseminated gonococcal infection developed antibodies reactive with gonococcal IgA1 protease; this incidence was no different than the one for uninfected controls (C. J. Lammel, M. S. Blake, W. D. Zollinger, E. W. Hook III, and G. F. Brooks, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 195, 1988). In contrast, the majority of patients with meningococcal disease or nasopharyngeal carriage had antibodies reactive with meningococcal IgA1 protease, and these antibodies were cross-reactive with the gonococcal IgA1 protease. Serum IgG reactive with meningococcal IgA1 protease inhibited the activity of both the meningococcal and gonococcal proteases.

MIRP. The pathogenic *Neisseria* spp. make several iron-regulated proteins under conditions of limited iron such as those that exist in the human host. One of these proteins, major iron-regulated protein (MIRP), is conserved among the pathogenic *Neisseria* spp. Patients with uncomplicated gonococcal infection, pelvic inflammatory disease, or disseminated infection had serum antibodies reactive with MIRP and showed moderate to high levels of reactive IgG and IgM and low levels of reactive IgA (21, 43). Reactive immunoglobulin levels were higher in patients with previous gonococcal infections and also increased when repeated infections occurred during the study. The results indicate that the iron-regulated protein is expressed in vivo. Patients with anti-MIRP serum immunoglobulins had repeated uncomplicated genital infections, and, hence, the serum antibodies were not protective for genital infection. A possible role for anti-MIRP antibodies in modifying or preventing gonococcal pelvic inflammatory disease is unknown.

Others. Several other antigens may be important in the humoral immune response to gonococcal infection. The outer membrane protein-macromolecular complex participates in the bactericidal activity of immune rabbit serum. We are not aware of published data on the human immune response to this complex. Patients with gonococcal infection make antibodies against proteins in the 46- to 48- and 54- to 58-kilodalton ranges and a variety of undefined higher-molecular-mass antigens (36); the role of antibodies against these antigens is undefined.

HUMAN MUCOSAL ANTIBODY RESPONSE IN GONOCOCCAL INFECTION

There are considerably fewer data on the genital antibody response in gonococcal infection than on the serum antibody response. This is particularly true with respect to specific gonococcal antigens.

Immunoglobulin Class

Studies of women have provided the most useful data on mucosal antibody response in gonococcal infection. Investigators commonly used fluorescent-antibody techniques and a laboratory strain of *N. gonorrhoeae* and looked for reactive IgA and IgG. In one study of six women with gonococcal cervicitis, the concentrations of vaginal-wash IgA were

higher than the concentrations in uninfected controls (44). The titers of antigonococcal IgA were high at the time of infection, often higher than in serum; after treatment, the IgA levels rapidly returned to normal (44, 60). One study of cervical secretions from 75 women with uncomplicated gonorrhea (UGC) showed antigonococcal IgG in 97% of the subjects, IgA in 95%, and IgM in 39%; antigonococcal IgG was found in the cervical secretions of 33% of 70 women who were not infected; no antigonococcal IgA and IgM were detected (42). Similar results showing the presence of more reactive IgG than IgA have been reported recently (28, 36). The predominant IgG subclass in vaginal secretions reactive with gonococcal antigens is IgG3 (34).

Men with UGC usually have urethral exudate antibodies reactive with gonococci. Antigonococcal IgA was found in exudates of 29 (83%) of 35 men with UGC (30); also, antigonococcal IgA was found in the exudates of 9 of 11 men with a first infection and 20 of 24 men with repeated UGC (31). Another study reported the presence of antigonococcal IgA in exudates from 98% of 132 men with UGC (41); reactive IgG and IgM were found in 90% and 49%, respectively. Of 100 men with nongonococcal urethritis or without urethritis, only one had antigonococcal IgA, but 26 had antigonococcal IgG. Following treatment, the levels of measurable antigonococcal IgA declined very rapidly; the IgG levels declined more slowly and could still be detected at 28 days after treatment.

Antigen Specificity

Study of the antigen specificity of antibodies in genital fluids has been difficult compared with analysis of antibody reactivity with whole gonococci.

Pili. *Pili, along with PI and PII, are the predominant* antigens in the genital immune response to genital gonococcal infection (28, 36).

In early gonococcal pilus vaccine studies it was found that vaginal fluid antibodies were reactive with pili, outer membranes, and LOS (60, 61). The pilus vaccine induced vaginal fluid antibodies that functioned to inhibit the pilus-mediated attachment of the homologous strain of *N. gonorrhoeae* (40, 58).

PI. PI is the primary antigen in both the genital IgG and IgA responses to gonococcal infection when tested with the infecting organisms of the patients (36). The genital anti-PI antibody level has not been quantitated, but it is qualitatively higher than the serum anti-PI antibody level.

PII. PII, along with PI and pili, is a major antigen in the female genital antibody response to gonococcal infection (36). We are not aware of any data on the genital anti-PII antibody response in men with gonorrhea. Whether the genital antibodies have a protective role in preventing or modifying gonococcal endocervical or urethral infection is unknown.

PIII. There is minimal if any measurable genital antibody response to PIII (36).

LOS. Although patients with genital infection make antibodies directed against gonococcal LOS, these antibodies are not as prominent in the immune response as are antibodies against PI, PII, and pili (28, 36).

H.8. One study in which vaginal fluid antibodies were studied for reactivity to a broad spectrum of gonococcal antigens did not demonstrate a genital antibody response to H.8 or to the broad band of reactivity that represents H.8 (36).

IgA1 Protease. Split products of IgA1 have been found in the genital secretions of women with gonorrhea, indicating that the gonococcal IgA1 protease is present and active during genital infection (6). To date, however, the enzyme has not been detected in genital secretions when a monoclonal antibody probe has been used; also, antibodies against IgA1 protease have not been found in genital secretions of women with gonorrhea (C. J. Lammel, M. S. Blake, and G. F. Brooks, unpublished observations).

MIRP. The reactive IgA and IgG levels in vaginal fluid were higher for pelvic inflammatory disease patients with no prior infections than for those with at least one prior infection (43). In contrast, the reactive IgA and IgG levels in vaginal fluid of UGC patients were higher for patients with prior infections than for those with no prior infections.

FUNCTIONAL IMMUNITY IN GONOCOCCAL INFECTION

Bactericidal and Phagocytic Systems

Extensive studies have been done on bactericidal and phagocytic systems in the immune response to gonococcal infection. Limited space precludes thorough review of the subject, and the reader is referred to other articles in this volume for additional information.

Gonococci isolated from patients with disseminated infection are resistant to the bactericidal action of most normal human sera and convalescent-phase sera (10, 53). This resistance is by virtue of blocking antibody directed against PIII and possibly other outer membrane antigens (7a, 47). Patients with a deficiency of one of the late-acting complement components also are at high risk for disseminated neisserial infection because their antibody-complement-mediated bactericidal systems are not functional (45). Antibodies also are opsonic for gonococci (10). A protective role for opsonization and phagocytosis is, however, not clear, because patients with late-acting complement component deficiency may have functional opsonization and phagocytosis and still have bacteremia.

The amount of complement present in the female genital tract is small (46) and probably will not allow complement-mediated bactericidal and opsonic systems to function there. Examination of a Gram stain of a genital exudate sample from an infected patient shows that gonococcal association with polymorphonuclear cells is important in the disease process. It is, however, not clear that the gonococcus-polymorphonuclear cell association in the genital tract is antibody mediated. Some PIIs mediate attachment of gonococci to polymorphonuclear cells, but it is not known whether human antibody can modify this process.

Preventing Attachment to Mucosal Cells

The pilus vaccine studies have provided evidence that anti-pilus antibody can prevent the attachment of homologous gonococci to mucosal cells (8, 59). The antigenic heterogeneity of gonococcal pili, however, may preclude a more broad-spectrum antibody-mediated prevention of gonococcal attachment to mucosal cells.

A monoclonal antibody directed against a PII can partially prevent PII-mediated attachment to eucaryotic cells. There are no data on the function of human anti-PII antibodies in modifying the PII-mediated attachment process, nor are there any data about human antibodies, other than anti-pilus antibodies, that modify attachment.

FURTHER QUESTIONS AND CONSIDERATIONS ABOUT IMMUNITY IN GONOCOCCAL INFECTION

Prevention of genital gonococcal infection and especially the major complication of pelvic inflammatory disease is an important objective. To date, however, there are no data on the humoral immune response to indicate that prevention of infection is likely.

Many studies have been oriented toward examination of antibody-complement-mediated bactericidal systems, but it is clear that the presence of serum bactericidal activity does not prevent uncomplicated gonococcal infection (9). It is not known whether the presence of serum antibody and bactericidal activity prevents or modifies gonococcal pelvic inflammatory disease.

Sera of patients may have high levels of antibody against pili, PI, PII, LOS, H.8, MIRP, and a variety of other gonococcal antigens. The patients also may have repeated uncomplicated genital gonococcal infection when these antibodies are present. It is, however, not known whether the presence of these antibodies prevents or modifies gonococcal pelvic inflammatory disease. Only one set of data from a small number of patients indicates that women do not have repeated gonococcal pelvic inflammatory disease with gonococci of the same PI type that caused their first infection (11).

There is insufficient understanding of genital antibody function in gonococcal infection to determine whether the antibody plays a role in modifying or preventing initial or repeated infection.

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Interaction of Complement with *Neisseria meningitidis* and *Neisseria gonorrhoeae*

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The importance of the complement system in host defense against systemic infection caused by neisseriae is emphasized by a number of clinical observations documenting the importance of antibody-dependent, complement-mediated serum bactericidal activity in protection from systemic meningococcal disease (17) and the well-recognized association between the resistance of gonococci to complement-dependent serum bactericidal activity and the development of disseminated gonococcal infection (46, 49). Moreover, individuals with inherited complement deficiencies have a markedly increased risk (ca. 6,000-fold) of acquiring systemic neisserial infections and are subject to recurrent episodes of systemic meningococcal and gonococcal infections (37, 43). The purpose of this report is to review the complement cascade with particular reference to its importance in host defense against bacterial diseases, to compare and contrast neisserial disease in complement-deficient and complement-sufficient individuals, to examine the molecular and functional basis for the particular importance of complement in host defense against these infections, and to examine the immunologic basis for prevention of neisserial disease in complement-deficient individuals.

THE COMPLEMENT CASCADE

Activation of complement by either the classical or the alternative pathway results in the formation of C3 convertases on the bacterial surface (13). These convertases cleave C3, initiate the alternative-pathway amplification loop, and participate in the formation of the C5 convertases. The C5 convertases in turn cleave C5, thereby initiating assembly of the membrane attack complex and its insertion in the membranes of susceptible bacteria (Fig. 1).

The reactions leading to the formation of the classical pathway C3 convertase, C4b2a, are initiated upon C1q binding to immunoglobulin M (IgM) or IgG during recognition of antigen by these antibodies. In this role, antibody serves to promote complement activation in a kinetically efficient manner and to direct its deposition to specific sites on the bacterial surface (15). The classical pathway can also be activated by the direct binding of C1q to certain bacterial substrates, for example, the lipid A moiety of endotoxin.

Alternative-pathway activation exhibits several fundamental differences from that of the classical pathway. First, antibody is not required, although it facilitates the activation process (39). Second, activation occurs continuously at a low rate which is tightly controlled by factors H and I, regulatory proteins present in plasma. Effective activation occurs when this control is subverted by the introduction of an activator surface (e.g., a bacterium), on which the binding of factor B to C3b is favored over the binding of the regulatory protein, factor H (13). Third, a component of the activation process, C3b, is also a product of activation, thereby generating a positive feedback loop (13). Consequently, C3b deposition mediated by either the alternative or the classical pathway is amplified by this loop. Amplification converts alternative-pathway activation from a kinetically inefficient to an efficient process. Fourth, in contrast to the classical pathway, in which antibody directs complement deposition to specific sites on the bacterial surface, alternative-pathway activation occurs randomly on the bacterial surface.

The C3 convertases are structurally and functionally homologous complexes which are bound covalently to the bacterial surface via ester or amide bonds (25). These linkages are formed upon rupture of an internal thiol ester bond in the α chain of both C4 and C3 during the activation of either of these molecules. Thus, the array of hydroxyl or amino groups available on the bacterial surface for the formation of ester or amide bonds with C4 or C3 is an important determinant of the outcome of the interaction between the complement system and the organism.

The central position of C3 at the convergence of the two activation pathways and at the head of the terminal complement pathway, as well as its role in initiating the alternative-pathway amplification loop, emphasizes the critical importance of this molecule and make it a logical point for regulation of complement activation. Regulation is achieved in the fluid phase by factors H and I (13) and on host cells by specific surface proteins which serve to distinguish self from nonself (1). In addition, specific chemical moieties can modulate C3 convertase function. An example of this type of control is sialic acid, a constituent of many glycoproteins present on human cells. Sialic acid enhances factor H binding to C3b approximately 100-fold compared with factor B. Hence, complement activation on the surface of these cells is effectively down-regulated, and the cell is protected from complement-mediated injury (12).

At a functional level, complement activation promotes an effective inflammatory response, participates in the elimination of immune complexes, helps to neutralize viruses, is capable of directly killing some gram-negative bacteria, plays an important role in opsonizing bacteria for ingestion and killing by phagocytic cells, and may play a role in the modulation of the immune response (Fig. 1).

NEISSERIAL DISEASE IN COMPLEMENT-DEFICIENT INDIVIDUALS

The frequency of inherited complement deficiency states in the general population is about 0.03% (16). Several studies have reported that the frequency of complement deficiencies among individuals with systemic neisserial disease ranges from 0 of 47 (<2%) to 3 of 20 (15%) (11, 38). This wide range is probably related to the ages of the patients in the studies, the relatively small number of patients studied, and a disproportionate genetic influence in relatively insular populations. The best estimate of the frequency of inherited complement deficiency states among patients with endemic neisserial disease is probably about 5 to 10%, although the

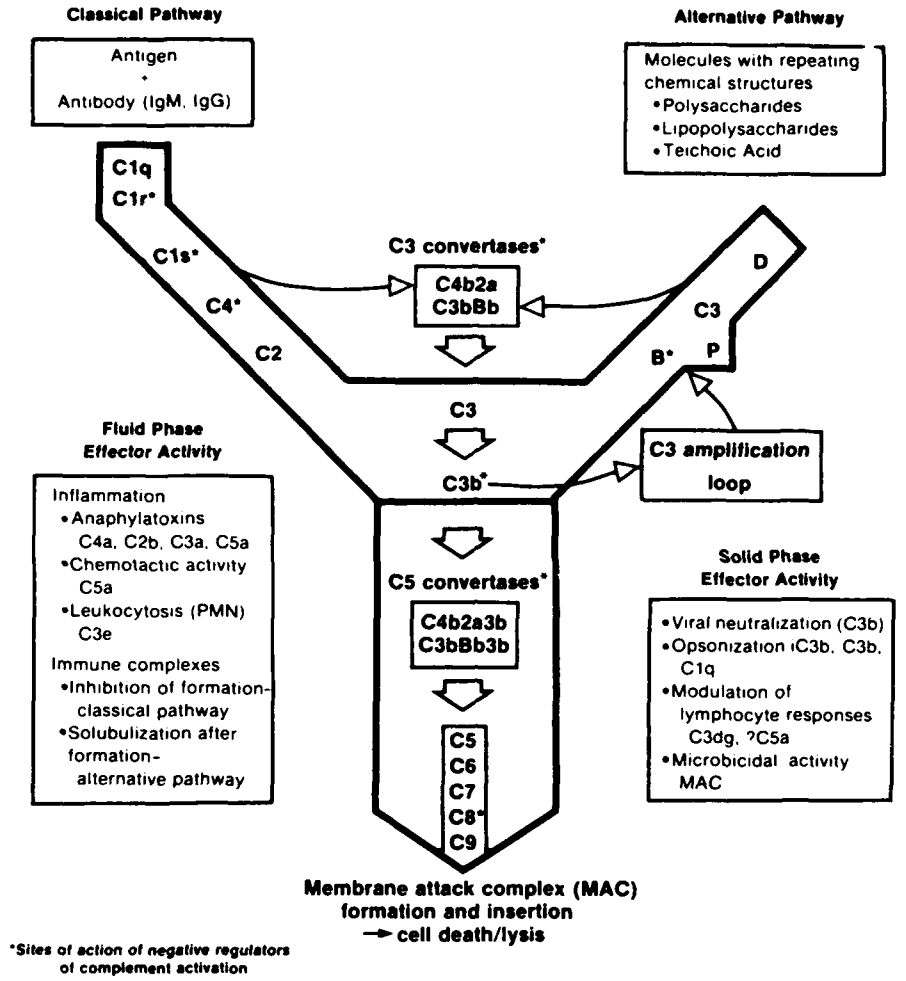

FIG. 1. The complement cascade. Within each pathway, the components are arranged in order of their activation and aligned opposite their functional and structural analog in the opposite pathway. Asterisks indicate sites of down-regulation of complement activity. Reproduced from P. Densen, *in* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett, ed., *Principles and Practice of Infectious Diseases*, 3rd ed., Churchill Livingstone, Ltd., Edinburgh, in press, with permission of the publisher.

likelihood of a complement deficiency increases dramatically (31%) among individuals who have had more than one episode of meningococcal infection (35). Thus, complement deficiency states occur approximately 150 to 300 times more frequently among individuals with systemic neisserial disease than in the noninfected general population.

Meningococcal disease is the most common infection experienced by complement-deficient individuals (43), although the frequency of infection differs among individuals with defects affecting different segments of the complement cascade. The low frequency of infection (20%) in individuals with a deficiency of C1, C4, or C2 compared with other component deficiencies (Table 1) is attributed to the presence of an intact alternative pathway in these patients (43). In contrast, although individuals with alternative-pathway defects can activate the classical pathway normally in the presence of specific antibody, the absence of antibody, coupled with a defective alternative pathway, leads to a profound abnormality in complement activation and serum bactericidal activity. Consequently, infection in such individuals might be expected to have dire consequences, a prediction borne out by statistics for properdin-deficient individuals (Table 1) (8, 47).

With the exception of C9 deficiency, individuals lacking one of the terminal complement components exhibit a striking susceptibility to systemic neisserial infection (37, 43). The basis for this association is the inability to express complement-dependent serum bactericidal activity. Support for this conclusion stems from the observation that the serum from C9-deficient individuals can kill meningococci, albeit at a lower rate than normal, a finding consistent with the fact that C9 is not absolutely required for complement-mediated lysis of erythrocytes and presumably accounts for the relative absence of meningococcal infections in these individuals (22).

The clinical pattern of meningococcal infection differs in complement-deficient and normal individuals (Table 1). In particular, in properdin deficiency, which is an X-linked trait, meningococcal disease occurs in males, and the first episode of infection usually occurs during the teenage years. The clinical course is frequently fulminant, and there is an associated high mortality rate; presumably, this is a consequence of low levels of specific antibodies, leading to an impaired capacity to utilize the classical pathway to opsonize bacteria and develop serum bactericidal activity (8, 47). The severity of the disease in these individuals contrasts with that in individuals with late complement component deficiencies, presumably because the former are unable to

TABLE 1. Comparison of meningococcal disease in normal, late complement component-deficient, and properdin-deficient individuals[a]

Type of host	Characteristics of individuals and infections[b]								Infecting serogroup		
	No. of homozygotes	No. with meningococcal disease	Frequency of infection (%)	Male/female ratio	Median age (yr) at 1st episode	Recurrence rate (%)	Relapse rate (%)	Mortality per episode (%)	No. of isolates	% B	% Y
Normal			0.0072	1.3:1	3	0.34	0.6	19	3,184	50.2	4.4
Late complement component deficient	195	124	64	2.8:1	17	46.2	4.7–5.8	1.6–2.7[c]	48	20.8	41.7
Properdin deficient	41	14–26	34–63	14:0–25:1	14–11.5	0 (<2.4)	0 (<2.4)	43–65	11	27.3	27.3

[a] Reproduced from P. Densen, *in* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett ed., *Principles and Practice of Infectious Diseases*, 3rd ed., Churchill Livingstone, Ltd., Edinburgh, in press, with permission of the publisher.

[b] Where a range is given, the first number refers to documented cases of meningococcal infection and the second number refers to documented plus probable and possible cases of meningococcal disease.

[c] The larger estimate includes two deaths in individuals with unconfirmed late complement component deficiency. The corresponding mortality rate per patient is 2.4 to 4.0%.

effectively recruit any C3-dependent host defense mechanisms, whereas the latter can express these activities to some degree. A striking finding in individuals with late complement component deficiencies compared with normal persons is the low mortality rate associated with meningococcal disease (43). This observation suggests that exuberant complement activation may contribute to the mortality rate in normal individuals and that this contribution is dependent in part upon the assembly of an intact membrane attack complex.

Uncommon meningococcal serogroups, in particular group Y strains, cause disease relatively more commonly among complement-deficient than normal individuals (43). This altered serogroup distribution appears to stem in part from the fact that group Y organisms are more serum sensitive but exhibit a more stringent requirement for elimination by phagocytic cells than group B strains (45). However, the neisseriae infecting complement-deficient patients are not more serum sensitive than the comparable organisms isolated from normal individuals. In particular, disseminated gonococcal infection in these individuals is caused by typical serum-resistant gonococci (42). This suggests that factors other than serum sensitivity and resistance (e.g., tissue invasiveness) contribute to the pathogenesis of neisserial infection.

MOLECULAR AND FUNCTIONAL BASIS FOR THE IMPORTANCE OF COMPLEMENT IN HOST DEFENSE AGAINST NEISSERIAE

A complete understanding of the interaction of complement with neisseriae requires the definition of the relevant antibody isotype (IgM versus IgG), its epitopic specificity, and the utilization of neisserial isolates with a defined surface composition (capsule, outer membrane protein, and lipooligosaccharide [LOS]). Ideally, such studies will examine the binding of multiple complement components as well as the functional activity of those bound components. At present, our knowledge of these variables is incomplete, so that the emerging picture of these interactions reveals a measure of complexity rather than detailed understanding. This section is an attempt to provide an understanding of these variables as they relate to complement activation by neisseriae at present.

A number of clinical studies have reported extensive complement consumption in individuals with fulminant meningococcal disease and have demonstrated a direct correlation between the presence of capsular polysaccharide in serum and the degree of complement consumption (2, 18, 24, 52). Complement consumption appears to occur via both the classical and alternative pathways, but the relatively normal C4 levels in association with the dramatic reduction in factor B concentration observed in these patients suggests that activation occurs primarily via the alternative pathway (24).

In vitro studies examining the pathway of complement activation by meningococci have confirmed that both group A and group B meningococci activate the classical pathway in normal serum (10). In contrast, only group A strains activate the alternative pathway (10, 28), a finding attributed to the fact that the group B capsular polysaccharide is a homopolymer of sialic acid (9, 29), which is known to inhibit alternative-pathway activation (12). This finding suggests that the absence of specific antibody to initiate classical-pathway activation, coupled with capsular sialic acid-mediated inhibition of alternative-pathway activity, may contribute to the prevalence of group B meningococcal disease in young children.

From the investigational standpoint, the capsular polysaccharides of group Y and W135 meningococci contain substituted sialic acid residues. Comparison of the effects of these defined variants upon alternative-pathway activity might shed further light on the mechanism by which sialic acid inhibits this activity (9, 29). Another aspect of this phenomenon is the observation of Zollinger and Mandrell that bactericidal titers of human antibody to group B meningococci are low when a human source of complement is used in the assay but are high when rabbit serum is used as the complement source (51). The explanation for this finding has not been rigorously examined, but it is probably due to the reported species specific capacity of sialic acid to inhibit alternative-pathway activity in humans but not rabbits.

Studies examining complement activation by gonococci incubated in chelated serum indicated that both pathways were utilized but that gonococcal serum bactericidal activity was associated primarily with activation of the classical pathway (26). Subsequently, we used complement-deficient serum samples from individuals not previously infected with neisseriae to reexamine this issue and to assess the contribution of both pathways to C3 fixation on serum-sensitive and serum-resistant strains (6). We found that the kinetics of gonococcal killing were identical in normal and in properdin-deficient serum containing an intact classical pathway. In contrast, achieving an equivalent degree of gonococcal killing in C2-deficient serum, which contained only an intact alternative pathway, took approximately three to four times

as long (6). Although different serum sources were used in these experiments, isotype-specific titers for gonococcal outer membrane proteins and LOSs did not differ among the serum sources used at the concentration employed in the assay. These data convincingly confirm the findings of Ingwer et al. (26) that activation of complement by and killing of gonococci in normal serum is mediated primarily through the classical pathway.

With respect to C3 deposition, our data indicated that substantially greater amounts of C3 were bound to serum-sensitive than resistant gonococci, although the pattern of deposition with respect to the pathway of activation was the same for both isolates (6). However, more interesting was the finding that more C3 was deposited on organisms incubated in normal serum than on those incubated in properdin-deficient serum, despite identical bactericidal kinetics in the two sera. Moreover, incubation of gonococci in C2-deficient serum for a period sufficiently long to allow a degree of killing equivalent to that in normal and properdin-deficient serum resulted in even greater C3 deposition. These data indicate that maximal C3 fixation to gonococci varies among sera, even though killing does not, and suggest that C3 binding to gonococci may occur at biochemically different sites on the organism surface and that some of these sites may be pathway specific (6).

Other investigators have observed that despite the presence of a hexosamine-containing LOS epitope for bactericidal IgM, gonococci exhibit a spectrum of sensitivity to lysis by normal human serum. Moreover, absorption of normal serum with these gonococci depleted alternative-pathway activity in proportion to the degree of their serum sensitivity and to their ability to bind purified properdin. These investigators suggested that classical pathway-initiated lysis of gonococci is variously augmented by the alternative pathway as a function of the ability of a given strain to bind properdin and that the titer at which a strain is lysed reflects this alternative-pathway augmentation (21, 27). Expressed differently, the greater the serum sensitivity of a specific organism, the greater the alternative-pathway augmentation. Thus, these results appear to contradict our findings that killing of an exquisitely serum-sensitive strain could be mediated solely by the classical pathway (6). Although the basis for this discrepancy is unresolved, one possibility is that the function of properdin in the complement cascade is believed to relate to its ability to bind to C3b (34) and to stabilize the alternative-pathway convertase (14), not to any intrinsic ability of this cationic protein to bind to a negatively charged target surface per se. This belief may require further scrutiny.

Serum-sensitive and resistant gonococci have been compared with respect to the outcome of a number of complement-mediated functions including opsonophagocytosis (C3b/iC3b), chemotaxis (C5a), and killing (C5b-9—the membrane attack complex) (5, 23, 31, 44). In each situation, complement activation was functionally effective when deposition occurred on sensitive but not on resistant gonococci. For example, sensitive strains were more rapidly ingested and killed by neutrophils in C8-deficient serum than resistant strains were (8.8% ± 3.4% versus 64.4% ± 7.7% survival at 30 min [$P < 0.005$]). Serum-sensitive isolates consumed and fixed C3 more rapidly and in greater amounts than did resistant strains (44). However, the difference in C3 consumption and fixation did not account for the difference in phagocytosis, because killing of sensitive strains was still greater than that of resistant strains under conditions of equal C3 fixation. More importantly, C3 bound to serum-resistant gonococci during incubation in normal human serum had no effect on promoting phagocytic ingestion and killing, since the slope of the plot of gonococcal survival versus C3 fixation was zero. In contrast, C3 fixation to sensitive strains made an important contribution to phagocytosis since ingestion and killing were significantly decreased when the serum was heated to inactivate complement (44).

Other investigators have demonstrated that complement activation by gonococci leads to the assembly of the membrane attack complex and its association with the gonococcal outer membrane (23, 31). C9 consumption and the number of C5b-9 complexes deposited on sensitive and resistant strains were equivalent, despite the different functional outcome resulting from this association. However, twice as many of these complexes could be removed from the resistant strain than from the sensitive strains by trypsin treatment, indicating that the complexes were inserted differently in the outer membranes of the two types of gonococci (31). In addition, the complexes were stably associated with distinctive proteins in the outer membrane of sensitive but not resistant strains (32). Moreover, treatment of resistant isolates with immune antiserum promoted both association of the membrane attack complex with these proteins and killing of the isolates (32). These data also demonstrate that resistant strains are not innately immune to complement-mediated attack.

Together, these data demonstrate the critical contribution of effective complement activation by serum-sensitive gonococci to multiple levels of host defense. Conversely, the ineffectiveness of this process in promoting neutrophil chemotaxis, phagocytosis, and serum bactericidal activity for serum-resistant gonococci is probably a significant factor in the pathogenesis of disseminated gonococcal infection and probably contributes to the relatively sparse discharge and lack of genital symptoms observed in this disease. The fact that resistant strains stimulate inadequate complement-mediated activities at multiple levels of the cascade (C3, C5a, and C5b-9) indicates that the basis for the impaired interaction between complement and these isolates must occur at a step preceding or involving C3 deposition. Moreover, these data suggest that immune antibody alters this process by directing complement deposition to specific sites on the organism surface (15).

Further support for this conclusion stems from our studies of the epitopic specificity of antibodies in normal and immune sera (4) and from experiments with monoclonal antibodies (33). In these studies we demonstrated a direct correlation between the titer of IgM anti-LOS for individual serum-sensitive strains and the complement-dependent chemotactic activity generated by the strain (4). Such a relationship was not demonstrated for serum-resistant gonococci, even though in some cases the individual IgM anti-LOS titer was higher than that for serum-sensitive strains. Nor was a correlation observed between complement-mediated activities and IgG anti-LOS titers or titers of either isotype against outer membrane proteins from any of the isolates. In convalescent-phase sera, IgM titers to outer membrane antigens of the homologous serum-resistant strain did not differ substantially from those in normal serum and recognized identical epitopes on Western immunoblots of purified LOS from the resistant strain. In contrast, in the convalescent-phase serum, IgG titers to outer membrane antigens were markedly elevated, were associated with effective complement-mediated activity, and recognized a unique LOS epitope not detected by normal IgG (4).

Joiner et al. (33) used a panel of monoclonal antibodies with overlapping isotype and subclass characteristics and sharing antigenic specificity for gonococcal outer membrane protein I to demonstrate that equal binding of these antibodies resulted in a range of gonococcal killing from 10 to 90%. This wide range in killing occurred despite the deposition of nearly equivalent numbers of C3 and C9 molecules on the organisms in the presence of these antibodies (33). In summary, both of these studies lend additional support to the importance of epitopic specificity in mediating effective complement disposition. This result is distinct from the effect of blocking antibodies (IgG specific for protein III on serum-resistant gonococci [40, 41] and IgA specific for capsular polysaccharide on meningococci [19, 20]), which compete with bactericidal antibody for binding sites on the organism. In the case of gonococci, blocking antibody also enhances complement deposition at sites which do not result in complement-dependent killing (30).

As a consequence of these findings, we examined the rate of cleavage of C3 covalently bound to serum-sensitive and resistant gonococci during incubation in normal human serum (C. McRill and P. Densen, unpublished data). As in our previous studies, more C3 was bound to sensitive than to resistant strains. Initial deposition of C3b on the sensitive strains was followed by its cleavage to iC3b, C3dg, and C3d, beginning within 5 min of incubation in serum. As C3b was cleaved to iC3b, factor B present as Bb bound to C3b was progressively lost from the cell surface, consistent with the decay of the alternative-pathway C3 convertase. In contrast, C3b deposition on resistant strains was not observed until after 10 min of incubation, and factor B, although bound by resistant strains, was neither cleaved to Bb nor shed from the cell surface. These studies demonstrate a difference in the rate of C3 cleavage on the two types of isolates as well as a difference in the interaction of factor B with C3b bound to them and suggest that the very rapid cleavage of C3b to iC3b on resistant strains may contribute to their resistance by preventing initiation of the assembly of the membrane attack complex (McRill and Densen, unpublished). The molecular basis underlying the difference in these reactivities requires further elucidation.

During the course of these studies, we sought to control the bacteriolytic effect of complement on these two types of isolates by using either C8-deficient serum or normal human serum immunochemically depleted of C8. We expected that these reagents would allow unencumbered C3 deposition on serum-sensitive strains but prevent C5b-9-mediated gonococcal lysis, thereby allowing sensitive strains to remain intact and morphologically analogous to serum-resistant strains. We were surprised to discover that although the total amount of C3 bound to serum-sensitive strains did not differ between complement-sufficient and deficient sera, factor B, present as Bb, was bound stably to C3 on sensitive gonococci incubated in complement-deficient but not in complement-sufficient serum (7). Reconstitution of the complement cascade by the addition of purified C8 to C8-deficient serum led to the loss of factor B and properdin previously bound to these organisms. Consistent with these observations was the finding of a delay in C3 cleavage on organisms incubated in deficient but not in sufficient serum. Additional studies demonstrated that this effect required C8 but not C9 in the nascent membrane attack complex, although the presence of C9 further enhanced factor B loss from the organisms (McRill and Densen, unpublished). When membrane disruption was prevented by depleting normal serum of lysozyme instead of C8, gonococcal killing and factor B loss occurred normally. These studies establish the existence of a novel feedback mechanism in which the assembly of the membrane attack complex promotes decay of the alternative-pathway C3 convertase, C3bBb:P, resulting in the release of factor B and properdin but not C3 from the organism surface (7). This effect does not require the gross morphologic disruption of the outer membrane. However, the extensive loss of outer membrane components containing endotoxin that occurs during incubation of neisseriae in complement-sufficient serum (7, 50), but not in deficient serum, may contribute to the higher mortality rate associated with meningococcal disease in normal individuals compared with that in patients with late complement component deficiencies (Table 1). In addition, the altered display of C3 cleavage products on organisms incubated in complement-deficient sera may lead to a difference in complement-dependent enhancement of immune responses.

IMMUNOLOGIC BASIS FOR PREVENTION OF NEISSERIAL DISEASE IN COMPLEMENT-DEFICIENT INDIVIDUALS

There is a sound theoretical and experimental basis for the use of capsular vaccines to prevent infection in individuals with inherited defects affecting either the classical or alternative pathway. In the former situation, specific antibody acts synergistically with properdin to enhance alternative-pathway activation and function (48), and postvaccination serum from these individuals demonstrates improved meningococcal killing (P. Densen, unpublished data). Similarly, we and others have shown that administration of the meningococcal vaccine to properdin-deficient individuals enhances the use of the classical pathway and the killing of meningococci (8, 48). Given the high mortality rate associated with meningococcal disease in these individuals, vaccination represents an important therapeutic strategy.

The theoretical basis for immunization of individuals with an inherited deficiency of one of the late complement components is less well established, since anti-capsular antibody cannot enhance serum bactericidal activity in individuals with a defective killing system. However, complement-dependent opsonization is unimpaired in patients with an inherited deficiency in one of the late complement components (36). We have shown that vaccination enhances the phagocytic elimination of meningococci from their serum (45). Nevertheless, phagocytic cells in the tissues and reticuloendothelial system do not seem to prevent recurrent neisserial infection in these patients. The reason for this apparent failure seems to be that the serum from unvaccinated and previously uninfected deficient patients, as well as serum from normal individuals, contains low levels of specific IgG anticapsular antibodies. Bactericidal antibody in these individuals is directed primarily at subcapsular antigens. Consequently, C3 is probably deposited at these sites rather than on the meningococcal capsule, resulting in impaired opsonization analogous to that described previously for pneumococci (3). Thus, the complement deficiency accounts for the susceptibility of these patients to meningococcal disease, but the associated lack of anticapsular antibody contributes to this susceptibility by impairing effective elimination of meningococci by phagocytes. Consequently, vaccination, by generating anticapsular antibody, should help protect these individuals by recruiting the phagocytic arm of host defense.

During recent studies of the immune response of late complement component-deficient individuals to meningo-

coccal infection, we observed that the levels of bactericidal antibody for group B meningococci did not differ among normal and complement-deficient individuals who had not experienced meningococcal infection (P. Densen, C. McRill, and M. Sanford, unpublished data). However, the serum samples from complement-deficient individuals with previous meningococcal infection contained significantly higher titers of bactericidal antibodies than did the serum samples of normal individuals with the same infection. This difference was independent of the number of infections, the time since the infection, the age of the patient, and the infecting meningococcal serogroup. Preliminary studies suggest that a likely target of this antibody is meningococcal LOS (Densen, et al., unpublished). These data suggest that there may be a subtle difference in antigen presentation in late complement component-deficient individuals. They also suggest the possible utility of LOS antigens as candidate vaccines in complement-sufficient individuals, particularly in the prevention of group B meningococcal disease.

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Plasmids of *Neisseria gonorrhoeae* and Other *Neisseria* Species

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All plasmids in bacteria are units which replicate independently of the bacterial chromosome, are generally less than 1/20 the size of the bacterial chromosome, and contain the information for self-replication. The 2.6-megadalton (MDa) cryptic gonococcal plasmids were first identified in the genus *Neisseria* in 1972 (13). Since cryptic plasmids had no measurable phenotype, they attracted little attention. The importance of plasmids in *Neisseria* spp. changed with the identification of β-lactamase plasmids in *N. gonorrhoeae* in 1976 (12). These plasmids encoded a β-lactamase which rendered the strains resistant to penicillin, the drug of choice for the therapy of gonorrhea at that time. As a result, β-lactamase plasmids had a great influence on the antibiotic treatment of gonorrhea. Since then, the indigenous 24.5-MDa conjugative gonococcal plasmids have been described and were shown to code for their own conjugal transfer, as well as mobilize the small gonococcal β-lactamase plasmids to other *N. gonorrhoeae* strains and *Neisseria* species (10, 16, 17, 21, 27, 32, 33). More recently, plasmids containing resistance determinants for tetracycline, sulfonamide, and other antibiotics have been described for various *Neisseria* species (14, 23–25, 28, 29, 37, 38).

Antibiotic resistance plasmids found in the genus *Neisseria* appear to be created either by direct acquisition of plasmids from other gram-negative bacteria or by the transposition of antibiotic resistance genes into indigenous neisserial plasmids. The β-lactamase and RSF1010-like plasmids are examples of direct acquisition of plasmids from other species (14, 38), whereas the 25.2 MDa TetM-containing plasmid is an example of a composite plasmid (28).

The presence of genetically related β-lactamase plasmids in both *N. gonorrhoeae* and *Haemophilus* spp. strongly suggested that plasmid exchange between these two genera had occurred in nature (4–6). Similarly, the presence of the RSF1010-like plasmids in *Neisseria* spp. indicated that plasmid exchange also occurred between the genus *Neisseria* and enteric bacteria (14, 37, 38). The 25.2-MDa plasmid has been transferred from *N. gonorrhoeae*, where it appears to have been created (28), into clinical strains of *N. meningitidis*, *Eikenella corrodens*, and *Kingella denitrificans* (22, 24, 35). Experimental studies have confirmed that the 25.2-MDa plasmid can be transferred to these species by conjugation under laboratory conditions.

In this review, I will describe the different types of plasmids currently found in the genus *Neisseria* and will indicate whether related plasmids are found in other genera as well. A summary of the plasmids discussed in this review is given in Table 1. Finding genetically related plasmids in both *Neisseria* spp. and other genera has helped to define the origin of many of the neisserial plasmids and has illustrated that the plasmids found in *Neisseria* spp. are directly related to the plasmid load carried by other bacteria which inhabit humans. On the basis of studies of the plasmids in *Neisseria* spp., one may conclude that gene and plasmid exchange is an ongoing process in nature and predict that the number of different plasmids and associated antibiotic resistance genes will increase in the genus over time.

NEISSERIA PLASMIDS

Cryptic Plasmids

The cryptic 2.6-MDa plasmids from *N. gonorrhoeae* were first described in 1972 (13). In an initial survey, we found that 96% of clinical isolates of *N. gonorrhoeae* carried this plasmid (34). However, more recently, it has been determined that all isolates of the proline-, citrulline-, and uracil-requiring auxotype are plasmid free (8). The reason for this is unclear; however, other auxotypes can also be plasmid free, although these are rare.

The 2.6-MDa cryptic plasmid is not correlated with the virulence of the gonococcal strains, pilus production, or the pilin protein, nor is it associated with the gonococcal outer membrane proteins PI, PII, or PIII, the immunoglobulin A1 proteases, the 37-kDa iron-regulated protein, or the receptor protein for the iron-retrieving siderophores (1, 2, 19). Thus, to date, this plasmid has no known function, even though the complete nucleotide sequence has been determined (26). Korch et al. (26) proposed a model for the genetic organization of the plasmid that predicted two transcriptional units, each composed of five compactly spaced genes. The promoter of one of the transcriptional units was shown to function in *Escherichia coli*. The products of three of the five genes in this operon were identified in minicell systems. Two genes in the other transcriptional unit were expressed in minicells when transcribed from an *E. coli* promoter.

It has been suggested that large segments of the 2.6-MDa plasmid can be integrated into the gonococcal chromosome in both plasmid-bearing and plasmid-free strains (19). In a few strains the entire 2.6-MDa plasmid may be integrated into the chromosome. A deletable region that contains repeated sequences has been identified in the plasmid. It is hypothesized that these repeats are involved in site-specific recombination with the chromosome and may explain why approximately 20% of the clinical isolates examined by Hagblom et al. carried a deleted variant of the cryptic plasmid (19).

Other *Neisseria* spp. also carry cryptic plasmids (11, 22, 30, 42). In one study, 69% of the plasmid-containing *N. meningitidis* strains, 33% of the plasmid-containing *N. lactamica* strains, 9% of the plasmid-containing *N. mucosa* strains, and 14% of the plasmid-containing *N. cinerea* strains were shown to carry plasmids of various sizes that hybridize with probes made from the 2.6-MDa plasmids (22). A few cryptic plasmids which do not share deoxyribonucleic acid (DNA) sequences with the 2.6-MDa plasmid have also been identified in strains of *N. meningitidis* (30, 42) and other commensal *Neisseria* spp. (10, 16). The cryptic plasmids, found in other *Neisseria* spp., do not occur as frequently as the 2.6-MDa gonococcal plasmid, and in general they have not been as well characterized as the 2.6-MDa gonococcal plasmid (11).

Gonococcal β-Lactamase Plasmids

The β-lactamase plasmids were first isolated in 1976 and were associated with strains from Africa and the Far East

TABLE 1. Plasmid classes found in the genus *Neisseria*

Plasmid class	Species	Reference(s)
Cryptic (2.6 MDa)	*N. gonorrhoeae*	13, 19, 26, 34
	N. cinerea[a]	22
	N. lactamica[a]	22
	N. meningitidis[a]	22
	N. mucosa[a]	22
β-Lactamase[b] (2.9, 3.05, 3.2, and 4.4 MDa)	*N. gonorrhoeae*	4–13, 33, 39, 41, 43
24.5-MDa conjugative	*N. gonorrhoeae*	11, 16, 17, 32–34
25.2-MDa conjugative[c]	*N. gonorrhoeae*	23, 25, 28
	N. meningitidis	24
RSF1010-like[d] (4.9, 7.0, 8.5, and 9.4 kb)	*N. meningitidis*	14, 38
	N. mucosa	29
	N. subflava	29
	N. sicca	29
Other (29 MDa)	*N. sicca*	Robledano et al., abstract

[a] These species were shown to carry plasmids of various sizes which hybridized with probes made from the 2.6-MDa plasmid.
[b] Related plasmids are found in the genus *Haemophilus* (4).
[c] Identical plasmids are found in clinical isolates of *K. denitrificans* and *Eikenella corrodens* (24).
[d] Related plasmids are found in enteric bacteria and *Eikenella corrodens* (37).

(12, 31). However, these plasmids are now endemic in isolates from North America, the Caribbean, and Europe, as well as Africa and Asia. They carry a TEM β-lactamase which hydrolyzes the cyclic amide bond in the β-lactam molecule and inactivates benzylpenicillin, ampicillin, and cephaloridine substrates but has low activity against isoazolyl penicillins such as oxacillin and methicillin (20). Originally, two plasmids were recognized at 4.4 and 3.2 MDa; each carried the TEM β-lactamase gene and 40% of Tn*A* transposon, which is the most frequent β-lactamase-encoding transposon in enteric bacteria (12, 20, 31). Recently, 2.9- and 3.05-MDa β-lactamase plasmids have been described; these appear to have been created by independent deletions of the 4.4-MDa plasmid (Fig. 1) (41, 43). The 2.9- and 3.05-MDa plasmids are not as common as the 4.4- or the 3.2-MDa plasmid.

The gonococcal β-lactamase plasmids are genetically related to small β-lactamase plasmids in various *Haemophilus* spp. (4–6, 31). These small β-lactamase plasmids from both genera are highly related and appear to represent a family of plasmids; they differ from each other by small deletions or insertions in either the Tn*A* region, which encodes the TEM β-lactamase, or the non-Tn*A* region (Fig. 1). The data indicate that these plasmids evolved from a single ancestral plasmid (4, 6, 7, 31). The small β-lactamase plasmids commonly found in *Haemophilus ducreyi* and *N. gonorrhoeae* contain 40 to 100% of the Tn*A* transposon sequences. These plasmids are less common in both *H. influenzae* and *H. parainfluenzae* (4–6, 31). The β-lactamase gene, which contains less than 100% of the Tn*A* transposon, is not transposable but gives rise to a functional transposon when linked to the left 2.4-kilobase (kb) *Bam*HI fragment from a complete Tn*A* transposon (15). These reconstructed transposons behaved like complete Tn*A* transposons because of their transposition frequencies. This suggested that the partial transposons contained the inverted repeats on the right side (IR-R) of the flanking sequence and must contain an intact fragment or a transposase (*tnpR*)-like gene. This supports the hypothesis that all the TEM β-lactamase genes originated from an intact Tn*A*-like transposon. DNA sequence analysis of the various plasmids has confirmed this hypothesis and is illustrated in Fig. 1 (5).

Gonococcal β-lactamase plasmids may be mobilized experimentally to *E. coli* or *Haemophilus* spp. by a variety of conjugative plasmids, including those from *E. coli*, *N. gonorrhoeae*, or *H. ducreyi* (4, 10, 16, 18, 27, 32, 33). However, initial attempts to transfer these β-lactamase plasmids into other *Neisseria* spp. met with only limited success (17). When introduced into the commensal *Neisseria* spp., the β-lactamase plasmids were very unstable and were quickly lost from most of the recipient strains. In a later study, Ikeda et al. (21) used the indigenous 24.5-MDa plasmid to transfer the 4.4-MDa β-lactamase plasmids from *N. gonorrhoeae* to strains of *N. meningitidis*.

Recently, using strains containing both the β-lactamase plasmid and the 25.2-MDa tetracycline resistance conjugative plasmid, we mobilized the 4.4- and 3.2-MDa β-lactamase plasmids into a variety of *Neisseria* spp., including *N. meningitidis* (36). A number of the transconjugants maintained the β-lactamase plasmids in the absence of selective antibiotic pressure. In all cases, the β-lactamase plasmid was associated with the 25.2-MDa plasmids in the transconjugants. We found that the *N. cinerea* transconjugants were exceptions to this generalization because when they were used as recipients, the β-lactamase plasmids were found without the 25.2-MDa plasmids (36). Recently, β-lactamase-producing *N. meningitidis* strains have been described (3, 9). In one case, the strain was isolated from a mixed culture with *N. gonorrhoeae* (9), and in the second case, it was not clear whether the β-lactamase enzyme was located on a plasmid or in the chromosome (3).

The small TEM β-lactamase plasmids have been transferred into *N. gonorrhoeae* and *E. coli* by transformation (31, 39). Sox et al. (39) showed that 25% of the plasmids in the gonococcal transformants had deletions, the most common of which was a 3.2-MDa plasmid. It is possible that the various gonococcal β-lactamase plasmids were created by deletions following transformation (39).

The DNA sequences of the gonococcal 4.4- and 3.2-MDa β-lactamase plasmids and the *H. ducreyi* 5.7- and 7.0-MDa β-lactamase plasmids have been determined (5). On the basis of these data and those of Yeung et al. (43) and Van Embden et al. (41), a model has been devised to account for the evolution of the small TEM β-lactamase plasmids (Fig. 1). The sequence of events hypothesized for this model is shown by the numbers 1 to 9 in Fig. 1 and explained in detail in the legend. Dickgiesser et al. (6) have suggested that the 1.8-kb insertion found in the 4.4- and 7.0-MDa plasmids, but not in the 5.7- or 3.4-MDa plasmids, is bounded by a short (200-base-pairs) terminal inverted repeat sequence and may be a bona fide insertion element.

GONOCOCCAL CONJUGATIVE PLASMIDS

24.5-MDa Conjugative Plasmids

The 24.5-MDa conjugative plasmid was first described in 1974 (11). It has been found in clinical strains of non-penicillinase-producing *N. gonorrhoeae* as well as in penicillinase-producing *N. gonorrhoeae* (11, 34), but not in any other clinical species. The prevalence of this plasmid varies geographically and temporally (34). In a recent survey of five geographical locations within the United States, the number of strains carrying the 24.5-MDa plasmid varied from 10 to

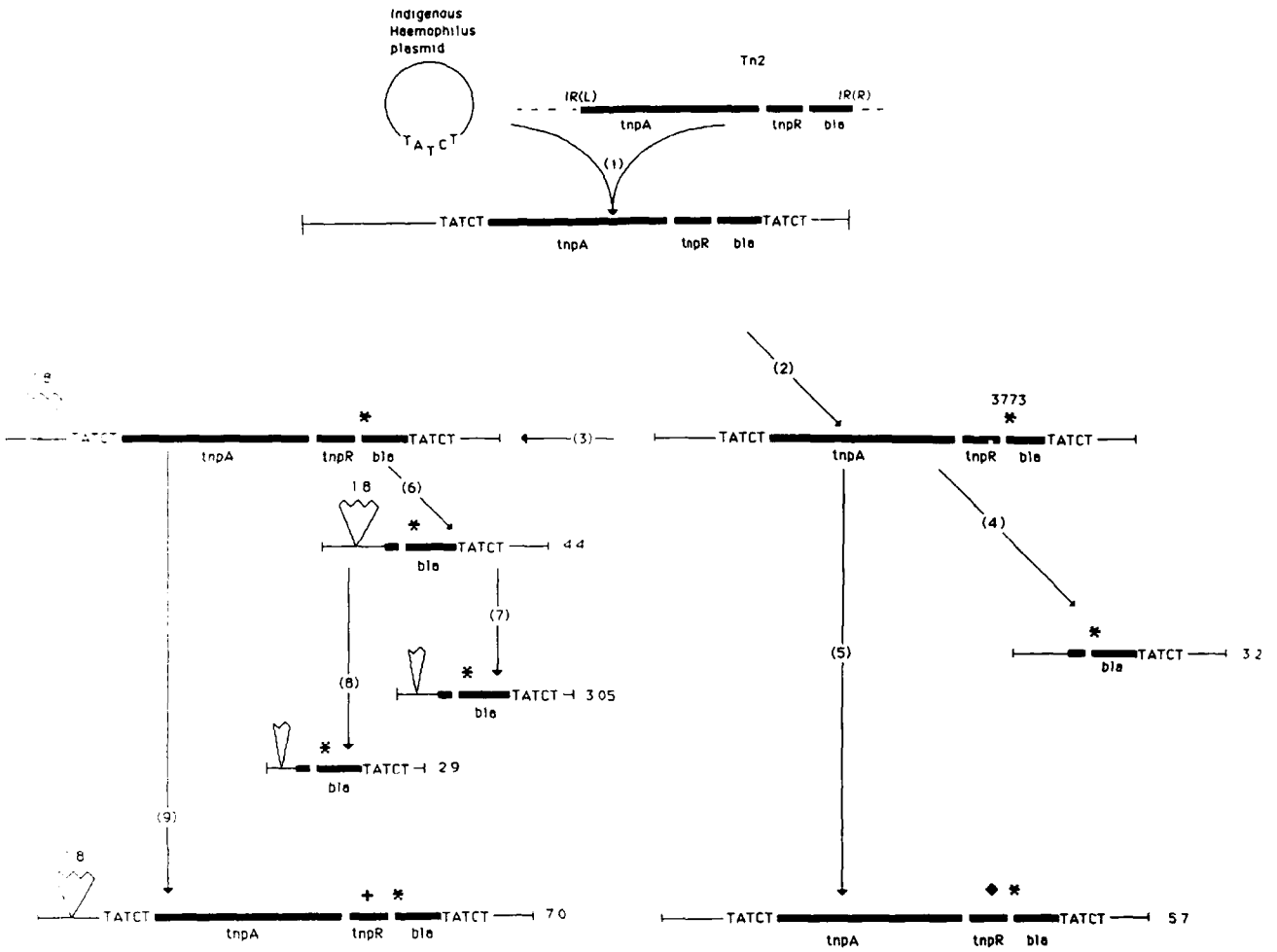

FIG. 1. Model for the creation of the small β-lactamase plasmids in *Neisseria* and *Haemophilus* spp. The events numbered in the figure are as follows. 1: Transposition of a Tn*2*-like transposon from an enteric plasmid onto an ancestral *Haemophilus* plasmid; 2: mutation in Tn*2* to replace a cytosine with a thymidine at base pair 3773 from the IR-L terminus of Tn*2* (*); 3: insertion of a 1.8-kb insertion sequence distal to IR-L; 4: deletion in the plasmid created in step 2 to produce the 3.2-MDa *N. gonorrhoeae* plasmid; 5: mutation from cytosine to thymidine (◆) in the noninsert plasmid made in step 2, to form the 5.7-MDa *H. ducreyi* plasmid; 6: independent deletion from the plasmid created in step 3, to produce the 4.4-MDa *N. gonorrhoeae* plasmid; 7: deletion of most of the 1.8-kb insert and a portion of the flanking sequences to create the 3.05-MDa *N. gonorrhoeae* "Toronto" plasmid; 8: another, separate, deletion in the same region of the plasmid to create the 2.9-MDa *N. gonorrhoeae* "Rio" plasmid; 9: mutation from guanosine to adenosine at position 3771 3′ (+) of the IR-L terminus to the form the 7.0-MDa *H. ducreyi* plasmid. The 4.4-, 3.05-, 2.9-, and 3.2-MDa plasmids have been isolated from strains of *N. gonorrhoeae*, whereas the 7.0- and 5.7-MDa plasmid have been isolated from strains of *H. ducreyi*. The other plasmids have not been isolated and are hypothetical. Data are summarized from references 5, 6, and 43.

53% (J. S. Knapp, J. M. Zenilman, R. Rice, M. C. Roberts, S. McIntire, and S. A. Morse, Sex. Transm. Dis., in press). In some cases, the 24.5-MDa plasmid is the only plasmid species present; however, it can coexist with the 2.6-MDa and the gonococcal β-lactamase plasmids (34).

The 24.5-MDa plasmid carries no detectable markers for antibiotic resistance, heavy metal, or ultraviolet resistance. However, it efficiently mobilizes itself and the smaller β-lactamase plasmids between strains of *N. gonorrhoeae* (10, 32, 33). When short mating times are used, only the β-lactamase plasmids are transferred to the recipient, whereas after overnight matings, many of the *N. gonorrhoeae* transconjugants carry both the β-lactamase and 24.5-MDa plasmids and can transfer both plasmids to other *N. gonorrhoeae* strains (32). The 24.5-MDa plasmid can also be used to mobilize the β-lactamase plasmids to other *Neiserria* spp. and *E. coli* (17, 21); however, it is not transferred to these other species. Its host range is very limited even under laboratory conditions, and other than *N. gonorrhoeae*, only certain strains of *N. cinerea* maintain it (17).

The structures of a limited number of 24.5-MDa plasmids have been determined; all had similar restriction patterns and shared 69 to 100% of their DNA sequences with two reference 24.5-MDa plasmids (34).

25.2-MDa TetM Conjugative Plasmids

The 25.2-MDa plasmid was first identified in a strain of *N. gonorrhoeae* isolated in 1982 (25). By 1985, a number of clinical isolates in North America and Europe carried this plasmid (23, 35, 28; M. C. Roberts, J. H. T. Wagenvoort, B. van Klingeren, and J. S. Knapp, Letter, Antimicrob. Agents Chemother. **32**:158, 1988). At present, the 25.2-MDa plasmid appears to be endemic in North America and has been found

in Great Britain and The Netherlands. Most of the strains in The Netherlands and a few from North America carry the 3.2-MDa β-lactamase plasmid in addition to the 25.5- and 2.6-MDa plasmids (25; Roberts et al., letter). Recently, the 24.5-MDa conjugative gonococcal plasmids have been shown to share >60% of their DNA sequences with the 25.2-MDa plasmids (28). These data have led to the hypothesis that the 25.2-MDa plasmid was formed by the transposition of the TetM determinant, encoding tetracycline resistance, onto the 24.5-MDa plasmid. The 25.2-MDa plasmids have characteristics which differ from those of the 24.5-MDa ancestral plasmids, but they have maintained the ability to move themselves and the β-lactamase plasmids to recipient strains (36). The 25.2-MDa plasmid has been associated only with the 3.2-MDa plasmid; therefore, it can be hypothesized that the 25.2-MDa plasmid was created in an *N. gonorrhoeae* population which carries the 3.2-MDa plasmid, such as in North America, Europe, Africa, or the Caribbean.

Naturally occurring strains of *N. meningitidis*, *K. denitrificans*, and *Eikenella corrodens* that carry the 25.2-MDa plasmid have been identified in both North America and Great Britain (24, 40); these species do not normally harbor the indigenous 24.5-MDa plasmid, and attempts to introduce this plasmid into strains of *N. meningitidis* and other *Neisseria* spp. have generally been unsuccessful (17). However, it has been shown experimentally that the 25.2-MDa plasmids may be transferred by conjugation, maintained, and transferred back to *N. gonorrhoeae* from *N. meningitidis*, as well as to the other *Neisseria* spp. tested (35).

Both the 24.5- and the 25.2-MDa plasmids can mobilize the gonococcal 4.4- and 3.2-MDa β-lactamase plasmids to a variety of bacterial species. However, when the 24.5-MDa plasmid is used to mobilize the β-lactamase plasmids, only *N. gonorrhoeae* and some of the *N. cinerea* transconjugants receive or maintain the 24.5-MDa plasmid (17). When the 25.2-MDa plasmid was used to mobilize the β-lactamase plasmids, all transconjugants, with the exception of some *N. cinerea* recipients, acquired and maintained the 25.2-MDa plasmid, whereas fewer transconjugants acquired the β-lactamase plasmid (36). Thus, the 25.2-MDa plasmid differs from the ancestral 24.5-MDa plasmid by its wide natural host range, while retaining its ability to mobilize the β-lactamase plasmids between strains. It is not known whether the ability of the 25.2-MDa plasmid to transfer to many bacterial species is due to the direct influence of the TetM genes, or whether the TetM determinant was inserted into the 24.5-MDa plasmid in such a way that it has altered or deleted DNA sequences that previously restricted the ability of the 24.5-MDa plasmid to be accepted or maintained in other bacterial species.

RSF1010-Like Plasmids

Recently, a new group of plasmids ranging in size from 4.9 to 9.4 MDa has been described for *N. meningitidis*, *N. mucosa*, *N. subflava*, and *N. sicca* (14, 29, 38). These plasmids are genetically related to the enteric plasmid RSF1010 which is an IncQ(P-4) plasmid that encodes streptomycin and sulfonamide resistance owing to the production of a streptomycin phosphotransferase and sulfonamide-resistant dihydropteroate synthetase (14, 38). A related plasmid has also been found in *Eikenella corrodens* (37). Some of these plasmids specify resistance to sulfonamide alone, whereas others specify resistance to penicillin, streptomycin, and sulfonamide (14, 29, 37, 38). In some cases, the relationship of the *Neisseria* plasmids to the ancestral RSF1010 plasmid has been based on DNA homology with the RSF1010 plasmid, including the antibiotic resistance region as well as regions outside the antibiotic resistance genes (29, 37, 38). In other cases, the relationship between the *Neisseria* plasmids has been based on more circumstantial data, such as similarities between the proteins encoded by the antibiotic resistance genes (14).

Plasmids encoding streptomycin, sulfonamide, and TEM-type β-lactamase resistance have been isolated from *N. mucosa*, *N. subflava*, *N. sicca*, and *Eikenella corrodens* (29, 37, 38), whereas plasmids specifying resistance to sulfonamide alone have been found in *N. meningitidis* strains (14). All of the multiresistance plasmids can be transferred by transformation and maintained in *E. coli* (29, 37, 38). The *N. meningitidis* sulfonamide resistance plasmid has been transferred by transformation and maintained in *N. gonorrhoeae* (14). This sulfonamide resistance plasmid may have been created (by the deletion of the streptomycin gene) from RSF1010 and then introduced into *N. meningitidis*. However, the *N. meningitidis* plasmid is larger than the RSF1010 ancestral plasmid, suggesting that it may have been created by a deletion of the streptomycin gene from RSF1010 followed by the insertion of other sequences or by the duplication of existing sequences (14).

The data obtained by several investigators suggest that the multiresistance plasmids carrying streptomycin, sulfonamide, and TEM β-lactamase (14, 38) were created by the transposition of Tn*A* onto RSF1010. These plasmids were subsequently spread, by mobilization or transformation, to other bacterial strains. The fact that the transposition of Tn*3* onto RSF1010-related plasmids occurs under laboratory conditions would support this hypothesis (20). However, there are differences between the naturally occurring plasmids and those created in the laboratory. The laboratory-created plasmids carry the whole Tn*3* transposon, whereas the naturally occurring plasmids do not (20). The possession of an incomplete Tn*3*-like segment by the multiresistance plasmids found in commensal *Neisseria* species is the only similarity between them and the gonococcal β-lactamase plasmids.

Other Plasmids

Robledano et al. (L. Robledano, M. J. Rivera, P. Madero, M. A. Marco, I. Otal, M. C. Aqudo and R. Gomez-Lus, Abstr. 5th Int. Congr. Chemother. 1987) reported the presence of a 29-MDa plasmid coding for ampicillin, tetracycline, streptomycin, kanamycin, neomycin and lividomycin resistance in a strain of *N. sicca*. The plasmid coded for two aminoglycoside-modifying enzymes, APH(3") and APH(3'). This plasmid was conjugally transferred to an *N. sicca* but not to an *E. coli* recipient strain, suggesting that it was not of enteric origin. The genetic relationship of this plasmid to other *Neisseria* plasmids has not been determined, and, as a result, the origin of this plasmid is unclear.

CONCLUSIONS

The 2.6- and 24.5-MDa plasmids have been found in isolates of *N. gonorrhoeae* preserved since the 1940s (34). In contrast, plasmids carrying antibiotic resistance genes are relatively new; the β-lactamase plasmids appeared in the 1970s (12) and the TetM-containing plasmids in the 1980s (25). Most of the antibiotic resistance genes that have spread into *Neisseria* spp. are associated with transposons. These determinants can either be introduced into new species on

plasmids capable of being maintained in the host, such as the broad-host-range enteric RSF1010-like plasmid or the *Haemophilus* β-lactamase plasmid, or be integrated into indigenous host plasmids as seen with the 25.2-MDa plasmid. In either case, once the R factor is created or introduced, it can then be spread by conjugation or mobilization to other strains, other species within the same genus, and other genera of bacteria. Transformation may play a role in plasmid spread between strains, because many of the plasmids discussed above have been experimentally transferred to various hosts by transformation (39). However, deletions often appear in these plasmids during transformation, and the frequency of transfer by transformation is usually significantly lower than the frequency of transfer by conjugation (39).

Gene spread has also occurred in the genus *Neisseria* as a result of the transfer of antibiotic resistance genes associated with the conjugative transposon, TetM (24). Clinical isolates of commensal *Neisseria* species have been isolated that have the TetM determinant integrated into their chromosome (24). The fact that commensal *Neisseria* spp. can acquire and maintain the 25.2-MDa plasmid if given the opportunity (35) suggests that the commensal *Neisseria* strains have not acquired the TetM determinant from *N. gonorrhoeae* but rather from other bacterial species. This would explain why in the laboratory the 25.2-MDa plasmid is stable in the commensal *Neisseria* spp. but all the natural clinical isolates carry the TetM determinant in the chromosome.

The RSF1010-like plasmids, in general, appear to be rather limited in their geographical distribution; this may be because very few investigators have examined commensal *Neisseria* spp. for antibiotic resistance genes. The recent observation that the RSF1010-like plasmids can carry not only the sulfonamide-resistant dihydropteroate synthetase and streptomycin phosphotransferase (normally associated with this plasmid) but also the TEM β-lactamase is of interest. The RSF1010-like plasmids are the first multiresistance plasmid group found in *Neisseria* spp. The versatility in the number of antibiotic resistance genes which the RSF1010-like plasmid can carry is unique among the neisserial plasmids. In addition, these plasmids are found in a number of different species. Since the RSF1010-like plasmid has already been found in *N. meningitidis*, it is very possible that this plasmid group will be disseminated to *N. gonorrhoeae*; therefore, more work to characterize this plasmid family should be done.

The number of plasmids carrying antibiotic resistance genes has increased in the genus *Neisseria* over the past 10 years. This increase is due not only to an increase in the number of strains carrying the previously described plasmids, but also to the identification of new plasmids carrying a number of different antibiotic resistance determinants in various commensal *Neisseria* spp. The antibiotic resistance genes currently found have all been identified in other bacterial genera, usually enteric species, and, with the exception of the TetM determinant, all plasmids carrying antibiotic resistance genes appear to have originated in other gram-negative bacteria. The TetM determinant appears to have originated in gram-positive streptococci (28). It might be anticipated that when the prevalence of plasmids and antibiotic resistance genes increases in other bacteria, especially the enteric species, it may lead to the transfer of these plasmids and antibiotic resistance genes to *Neisseria* spp. It is important, therefore, to continue screening isolates of both pathogenic and nonpathogenic *Neisseria* spp. for antibiotic resistance and then to determine whether the resistance is due to the acquisition of new genetic material or to chromosomal mutation. The commensal species have been shown to carry multiresistance plasmids and may act as reservoirs for plasmids and antibiotic resistance genes that might be transferred to the pathogenic *Neisseria* species at a later date. Antimicrobial therapy for gonorrhea and meningitis may be more complicated in the future if these multiresistance plasmids become established in the pathogenic *Neisseria* spp.

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Gene Transfer in *Neisseria gonorrhoeae*

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The ability of bacteria to exchange deoxyribonucleic acid (DNA) endows these organisms with greater genetic variability and increased capability to adapt to changing environments. Many bacteria have evolved transformation and conjugation systems to effect this exchange, whereas others achieve it through the action of bacteriophages. In *Neisseria gonorrhoeae* no transducing bacteriophages have been identified, but conjugation and transformation both occur (10). Gonococci are extremely autolytic and therefore release DNA to neighboring cells (19). Transformation with chromosomal markers has been demonstrated between strains in laboratory-grown mixed cultures (32); similar transformation probably occurs in nature. Quite recently, antigenic and phase variation of gonococcal pili has been shown to be due in large part to release of DNA from autolyzing cells, with subsequent transformation of other competent cells in the population by variant *pil* sequences (32a; C. Haas and T. Meyer, personal communication). Gonococcal transformation has been studied extensively and has proven useful in the construction of isogenic strains for mapping antibiotic resistance genes and biosynthetic auxotrophs, and in studies of molecules implicated in the virulence of this organism (10, 34). Conjugation in *N. gonorrhoeae* is important because it results in mobilization of antibiotic resistance plasmids, but chromosomal genes cannot be transferred by conjugation (3, 10, 27, 31, 37, 44). Thus, in the laboratory and in nature, transformation is the primary means of transfer of chromosomal genes.

In this brief review, mechanisms of gene exchange will be emphasized, and some speculative comments on possible future developments are included.

TRANSFORMATION

Many bacteria are competent for the adsorption and uptake of free DNA (36). Competence is defined as the ability to take up DNA into a deoxyribonuclease-resistant form, and it usually occurs only under special environmental conditions or in certain growth phases (7, 39). *N. gonorrhoeae* is unique in that it is constitutively competent at all phases of growth. The uptake of both plasmid and chromosomal DNA by gonococci is dependent only on the presence of glucose, as well as monovalent or divalent cations, in the medium (6). Competence is restricted, however, to piliated organisms (38) or to certain organisms that produce pilin subunits (Haas and Meyer, personal communication). Loss of pili occurs readily in vitro, and most nonpiliated variants that have been studied are reduced in competence by at least a factor of 10^4 compared with piliated cells (10, 21, 38, 39).

The first step in transformation is the binding of DNA to the cell surface. Although gonococci bind both homologous and heterologous DNA, only homologous DNA is taken up into a deoxyribonuclease-resistant form (14). The entry of plasmid and chromosomal DNAs appears to share an initial binding step at the cell surface, since uptake of plasmid DNA can be competitively inhibited by the presence of exogenous chromosomal DNA (18). Putative surface receptors which may be involved in the specific recognition and binding of homologous DNA have not been identified yet. The very close correlation between piliation and competence suggests that pili might be involved in DNA uptake, but there is no evidence that pili bind DNA in vitro (26). Moreover, neither purified pili nor anti-pilus antibodies block transformation by limiting concentrations of DNA (26; G. Biswas, N. Guerina, C. Brinton, and P. F. Sparling, unpublished data).

To better understand mechanisms of transformation, we have studied transformation-deficient mutants. Two mutants designated *dud* (DNA uptake deficient) were identified following chemical mutagenesis and are normally piliated as seen under the electron microscope but are unable to take up DNA (6a). This indicates that pili may not be sufficient for specifying DNA uptake in gonococci, although data are insufficient to rule out a minor structural change in pilin in these strains. Interestingly, Klimpel and Clark recently reported that phase variation of pili is correlated with altered expression of several cytoplasmic and membrane-bound proteins (23). It is possible that one or more of these are competence related. Further study of the *dud* lesion may aid in the identification of proteins involved in DNA uptake.

In addition to the differences in internalization of homologous and heterologous DNA, some gonococcal DNA sequences are internalized more efficiently than others. Graves et al. studied the uptake of fragments of pFA10, a cointegrate plasmid composed of the heterologous penicillin resistance plasmid pFA3 and the gonococcal cryptic plasmid pFA1 (18). Single *Msp*I or *Taq*I restriction endonuclease fragments derived from the pFA1 portion of pFA10 were preferentially internalized, suggesting the presence of specific uptake sequences, as has been shown in *Haemophilus influenzae* (12, 16, 35). Burnstein et al. noted that two uptake-preferred fragments of pFA1 shared a 10-base-pair (bp) sequence (5′-GATGCTCTGT-3′) (9). However, this sequence was not sufficient to specify high-frequency uptake of a pBR322 derivative into which it was inserted. More recently, Goodman and Scocca identified chromosomal DNA fragments of *N. gonorrhoeae* which were able to competitively inhibit transformation (17). A 10-bp sequence (5′-GCCGTCTGAA-3′) was common to such fragments and was sufficient to competitively inhibit transformation when cloned into the noncompeting pBR322. Thus, similar to the 11-bp recognition sequence responsible for DNA uptake in *H. influenzae*, there is a short DNA sequence in *N. gonorrhoeae* which appears to be involved in recognition of gonococcal DNA. It is interesting that the gonococcal recognition sequences identified by Goodman and Scocca are contained within the transcriptional termination sequences of the *opaE1* and immunoglobulin A protease genes, as well as three unidentified genes. This may be a general phenomenon and may suggest a mechanism by which this compo-

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nent of the DNA uptake apparatus has evolved and is preserved.

Transforming chromosomal DNA enters the gonococcal cell as a double-stranded molecule and remains principally double stranded inside the cell until homologous recombination results in transformation (8). There is no eclipse of biological activity of donor DNA during entry or for 60 min thereafter, which strongly favors the hypothesis that the donor DNA retains the double-stranded form, since single-stranded donor DNA has very little activity. Moreover, newly introduced DNA fractionates with labeled double-stranded DNA molecules when subjected to CsCl isopycnic centrifugation. In these respects also the gonococcal transformation system is similar to *H. influenzae* (28, 45). It is not known whether DNA enters through specialized surface structures similar to the transformasomes reported in *H. influenzae* (1, 20).

When circular plasmid DNA from an isogenic strain is used in transformation, a substantial proportion of the transformants contain plasmids which are either larger or smaller than the transforming plasmid. Studies with the hybrid plasmid pFA14 have helped to elucidate the mechanism by which entering plasmid DNA becomes stabilized (6). This plasmid is a 42-kilobase hybrid composed of the β-lactamase (Ap^r) plasmid pFA3 and the gonococcal conjugative plasmid pFA2. Circular pFA14 ordinarily is cleaved to many small fragments during entry, and plasmids recovered from rare transformants invariably contain deletions. If the recipient contains the homologous replicon pFA2, however, pFA14 results in abundant Ap^r transformants, and no deletions are observed among plasmids recovered from transformants. The ability of pFA14 to transform pFA2-containing recipients is thought to be due to marker rescue of fragmented pFA14 by the resident pFA2. Similarly, pFA10 (pFA3-pFA1) transforms pFA1-containing strains at least 10-fold more efficiently than it transforms plasmid-free recipients (5). Thus, following linearization of plasmid DNA during entry and random cleavage to smaller fragments, the fragments are either religated to form deleted plasmids (an infrequent event) or rescued by homologous recombination with resident (plasmid) DNA (4, 6). This probably requires the gonococcal Rec function, since Rec strains are nontransformable with plasmid (or chromosomal) DNA (24; M. Koomey, personal communication). The cleavage of entering DNA is not mediated by a restriction endonuclease, since these results were obtained in entirely isogenic backgrounds.

N. gonorrhoeae produces at least five different restriction enzymes and eleven different methylases (13, 25). A screening of 30 gonococcal isolates revealed that all but one had methylase activity, and about half produced detectable restriction enzymes (41). These restriction modification systems probably play an integral role in the survival of transforming DNA, although methylation does not affect the uptake of DNA into the cell (9, 18). Stein et al. showed that a plasmid (pFT180) isolated from a nonmethylating strain (WR302) was unable to transform an *Ngo*II-producing strain (Pgh3-2), while pFT180 isolated from Pgh3-2 transformed Pgh3-2 very well (40). Prior in vitro methylation with *Hae*III methylase of pFT180 isolated from WR302 enabled this plasmid to transform Pgh3-2 with more than 1,000-fold greater efficiency. Thus, as with *Escherichia coli* and probably many other bacteria, *N. gonorrhoeae* appears to control the influx of DNA in part by recognizing methylation patterns and restricting those deemed to be foreign.

CONJUGATION

Plasmids such as pFA3, which are not conjugally proficient, can be mobilized for conjugal transfer by 36-kilobase plasmids such as pFA2 (2, 3, 10, 22, 30, 37). Gonococcal conjugation mediated by pFA2 is extremely efficient, since under optimal conditions nearly 100% of conjugation donors may transfer the Ap^r plasmid pFA3 to recipients in a 90-min filter mating (3). This suggests that the pFA2-mediated system is naturally derepressed. It does not appear to mobilize chromosomal markers, however. Host factors are critical in conjugal efficiency. Maximal conjugation efficiency was observed when derivatives of the gonococcal strain F62 were used as recipients. Matings between unrelated gonococcal strains often resulted in transfer efficiencies 1,000-fold lower than those involving isogenic strains. This does not appear to be an effect of restriction modification systems, since transfer of the R-group plasmid pFT6 was relatively unaffected by restriction modification differences between the conjugation donor and recipient (40, 41). It is possible that the reduced efficiency of interstrain conjugation is due to differences in surface components required for stable mating pair formation.

Little is known about the physical aspects of gonococcal conjugation. Cell-to-cell contact is required; this contact does not appear to be pilus mediated, since mating pair formation is unaffected by the state of piliation (15). The presence of the 28-kilodalton form of outer membrane protein PII in either parent reduced the conjugal transfer of pFA3 (3). Other outer membrane components, which may be intimately involved in mating pair formation or in actual DNA transfer, have not been identified. In *E. coli* conjugation, DNA is transferred as a single-stranded molecule. That this may also be so in *N. gonorrhoeae* is supported by the similar mating efficiencies between isogenic strains or strains differing with respect to their restriction modification systems. Recipient restriction endonucleases would be expected to have little effect on entering single-stranded DNA.

TRANSFER OF CLONED GENES INTO GONOCOCCI

As more gonococcal genes are cloned and as techniques for the deliberate mutation of these genes in *E. coli* are developed, it becomes increasingly important to be able to return this DNA to *N. gonorrhoeae* for examination of gene function. Few studies have been published with the transformation of *N. gonorrhoeae* with gonococcal DNA cloned into *E. coli*. The first reports of success were by Koomey and colleagues, who used insertions of the *bla* (β-lactamase) gene to inactivate the gonococcal IgA protease (24a) and the *recA* genes (24); selection for Ap^r transformants resulted in allelic replacement of the wild-type gene by the mutated, cloned gene. Stein et al. used the shuttle vector pLES2 to clone a gonococcal proline-biosynthetic gene in *E. coli*; this construction transforms gonococci efficiently and exists stably, either integrated into the host chromosome or as an autonomously replicating plasmid (42, 43; G. Biswas and P. F. Sparling, unpublished data).

Attempts to elucidate the functions of gonococcal genes are aided by recent mutagenesis techniques. Since transposons have not been demonstrated in gonococci, a system for introducing transposon insertions into gonococcal genes cloned into *E. coli* was developed by Seifert et al. (33). A gene encoding chloramphenicol acetyltransferase (*cat*) was inserted into a Tn*3* derivative lacking sequences coding for the *trans*-acting factors necessary to transpose. This mini-

transposon (mTn*3-cat*) can be induced to insert into cloned gonococcal genes by providing transposase and resolvase functions in *E. coli*, in a vector constructed so that the great majority of viable transposon insertions occur within the cloned DNA. The resulting *cat*-marked DNA is then returned to the gonococcus by transformation and selection of chloramphenicol-resistant (Cm^r) transformants. Transposon-mutagenized DNA replaces the wild-type chromosomal allele by homologous recombination. This allows the construction of isogenic strains, differing only in the functional presence of the gene of interest. This system, termed shuttle mutagenesis, is being used with success in several laboratories. The only other available system at present to perform similar experiments involves the use of the β-lactamase (*bla*) gene of Tn*3* (24, 24a). However, *cat* results in significant chloramphenicol resistance when present in single copy in the gonococcal chromosome, whereas *bla* results in very low ampicillin or penicillin resistance; thus, mTn*3-cat* is preferred.

We have examined the fate of cloned gonococcal DNA when a recombinant plasmid is introduced into gonococci from *E. coli* by either transformation or conjugation. For these studies, we placed an mTn*3-cat* transposon adjacent to a portion of a cloned gonococcal PIA gene (11) in the shuttle vector pLES2. This plasmid (pUNCH102) can be transformed as an intact (circular) molecule from *E. coli* into gonococci or mobilized by incompatibility group P (IncP) plasmids such as pRK2013 for transfer into gonococci (our unpublished data). Similar conjugation experiments have been performed by others (29). The efficiency of gonococcal transformation by pUNCH102 prepared in *E. coli* was strain dependent. The highest efficiency of transformation was obtained with strain F62, whereas with strains FA19 and MS11 it was much lower. It is possible that this was due to differences in restriction endonucleases in these strains. Furthermore, all Cm^r transformants contained pUNCH102 integrated into the host chromosome, including at least a portion of the pLES2 vector (G. Biswas, N. Carbonetti, and P. F. Sparling, unpublished data). The efficiency of pRK2013-mobilized transfer of pUNCH102 from *E. coli* also was recipient strain dependent, with F62 again being the most efficient. As many as 10% of donor *E. coli* cells transferred pUNCH102 conjugally to F62, whereas transformation resulted in much lower transfer frequencies (ca. 10^{-6}). Most Cm^r transconjugants contained autonomously replicating plasmids identical in size to pUNCH102, and there was little evidence for integration of insert or vector sequences into the chromosome. By probing Southern transfers of restriction digests of the transconjugants either with an oligonucleotide specific for PIA or with a probe for *cat*, we searched for evidence of exchange of DNA between the chromosome and the plasmid, but found no evidence of allelic exchange between plasmid and chromosome (Biswas et al., unpublished data). Cloned gonococcal proline biosynthesis genes also have been returned to gonococcal cells by conjugation, and this therefore appears to be an attractive means of avoiding the problems caused by host restriction modification. Since conjugal transfer is very efficient, and insert DNA appears to be relatively stable on the introduced recombinant plasmid, this system may prove useful in cloning genes by mobilization of a whole library from *E. coli* into gonococci. This may be particularly useful if there is a positive selection for the gene of interest.

In addition, IncP plasmid-mediated conjugation may lead to the development of transposon mutagenesis systems within *N. gonorrhoeae*. Mutagenesis with transposons on suicide vectors via IncP conjugation has been successful in other gram-negative bacteria. A similar gonococcal system may be possible by using the easily selectable *cat* marker. We are constructing a chloramphenicol-resistant derivative of the transposon Tn*5* (Tn*5-cat*) placed on a plasmid which can be mobilized into gonococci by an IncP plasmid, but which is unable to replicate in gonococci (N. Carbonetti, V. Simnad, and P. F. Sparling, unpublished data). Presumably, Cm^r survivors would contain Tn*5-cat* transposed from the introduced plasmid into the host chromosome. It is not yet clear that this strategy will work in gonococci, but the effort is worthwhile because of the quantum leap such a system would provide for students of gonococcal biology.

When different fragments of gonococcal DNA marked by mTn*3-cat* prepared in *E. coli* were linearized and returned to gonococci by transformation, Cm^r transformants were obtained at highly variable frequencies, ranging from $<10^{-9}$ to 10^{-4} (H. Seifert, personal communication). Whether this is due to differences in gonococcal DNA uptake sequences, cleavage by restriction enzymes, or other factors remains to be shown. It will be interesting to see whether the addition of the putative gonococcal uptake sequence identified by Goodman and Scocca (17) to inefficiently transforming *cat*-marked DNA has any effect on its uptake.

CONCLUSIONS

Transformation is the primary means of transfer of chromosomal genes in gonococci. Competence is generally restricted to piliated organisms. Evidence for direct involvement of pili in transformation is principally circumstantial, however. Future studies including the *dud* mutants should lead to identification of the protein(s) involved in DNA uptake.

The uptake of DNA in the gonococcus is specific to homologous DNA or to DNA that contains the requisite structure for uptake by competent gonococci. A 10-bp DNA recognition sequence was identified recently (17) on the basis of the ability of DNA fragments to compete for transformation by gonococcal chromosomal DNA. It remains to be determined whether this 10-bp sequence confers uptake specificity. If the 10-bp recognition sequence is shown to increase uptake and transformation by various cloned gonococcal genes, future work may be facilitated by developing a transposon that contains the 10-bp recognition sequence as well as an easily selectable antibiotic resistance marker.

During entry, most circular plasmid DNA is randomly cleaved and enters cells as double-stranded molecules. Subsequently the linear molecules are either religated to form plasmids or rescued by homologous recombination. In view of the fact that gonococci produce a variety of methylases and restriction enzymes, plasmid transformation in gonococci is susceptible to restriction and modification systems.

Conjugation in gonococci results in very efficient mobilization of plasmids between gonococci, but chromosomal genes cannot be transferred between gonococci. Recent work suggests that the IncP conjugal system mobilizes certain plasmids efficiently from *E. coli* into gonococci, which may prove useful in devising strategies to clone gonococcal genes by first establishing them in recombinant libraries and then mobilizing the library into gonococci, with selection for the trait of interest.

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Cellular Immune Responses during Gonococcal and Meningococcal Infections

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The genus *Neisseria* contains two species of clinical importance: *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Although these two microorganisms are closely related genetically, the diseases they produce differ greatly. *N. gonorrhoeae* is the etiologic agent of gonorrhea, currently among the most common of the classical sexually transmitted diseases, whereas *N. meningitidis* is the causative agent of meningococcal meningitis. Although these *Neisseria* species have been known for over a century and there are effective antibiotic therapies for both of these diseases, the morbidity and mortality associated with them remain significant. For example, the pediatric mortality of meningococcal infection places it among the top 10 leading causes of death of children in North America. Similarly, the number of cases of gonorrhea reported to the Centers for Disease Control has averaged about three-quarters of a million per year for the past several years.

Despite a number of studies, the immunological mechanisms responsible for providing antigonococcal protection have been completely elucidated. In part, this is due to the lack of an animal model for studying gonococcal infection. Equally important, however, is the fact that most studies have concentrated on the systemic immune response to gonococcal infection (19, 25, 32, 37, 38), whereas the initiation of the infection occurs locally in a site apparently isolated from these circulating immune modalities. Thus, to completely understand antigonococcal immunity, it is necessary to study the local immune responses available at or near mucosal surfaces in the male and female reproductive tracts.

Serum antibodies specific for a variety of surface antigens have been described in both meningococcal and gonococcal infections, and in some cases the presence of these antibodies can be correlated with resistance to infection. It has been proposed that these antibodies could be involved in complement-mediated bacteriolysis or opsonization of the microorganisms or both. Although these mechanisms may be important in providing systemic immunity, sites where the *Neisseria* spp. are commonly found (the nasopharynx and reproductive tract) are sparsely populated with phagocytes and are deficient in many of the components of complement.

Recently, an increasing number of reports suggest that beside macrophage-dependent activities, cell-mediated responses such as cytotoxic T lymphocytes, natural killer (NK) cells, and antibody-dependent cell-mediated cytotoxicity (ADCC) might also have a role in the host defense against bacterial infection. A number of bacteria, including both gonococci and meningococci, make initial contact with the host at the mucosal level. It would follow that immunosurveillance mechanisms should be especially active at these anatomical sites. It is striking that large granular lymphocytes, the main effector cells of NK cell activity and ADCC, are present in large numbers in the epithelium and the lamina propria of musocal tissue and are able to exert their functional activity at this level. NK cells and ADCC appear to be manifestations of different functions of the same lymphocyte, and it has been suggested that intestinal large granular lymphocytes may play a role in the antibacterial secretory immunoglobulin A (sIgA)-dependent ADCC (13). A second, well characterized class of lymphocytes, the cytotoxic T lymphocytes, also may exist in musocal sites and may be active in providing antibacterial immunity. Most of these studies of cytotoxic mechanisms have focused on one or another of these distinct cell types, which have clear differences in the way they recognize target cells. It is the purpose of this paper to review recent evidence on cell-mediated immune (CMI) responses to pathogenic *Neisseria* spp. and to reevaluate their role in the development of antibacterial immunity.

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HOST CELLULAR DEFENSES IN GONOCOCCAL INFECTION

CMI Response to Gonococcal Infections

A few early reports in the literature suggest that a CMI response occurs in patients with gonococcal infection (1, 2, 5, 12, 31). An initial report of a delayed-type hypersensitivity response to gonococcal infection was published by Teague and Torrey (36). During the 1930s and 1940s, delayed-type hypersensitivity was demonstrated in individuals infected with gonococci by using a variety of cellular fractions and culture filtrates (1, 2, 5). Much of this work was done with the intent of developing a better method to diagnose the disease. Results of these studies indicated that most infected patients gave a positive result and up to 86% of normal individuals had a negative response (5). In the 1970s, interest was generated in the CMI response to gonococcal infection. Reports of lymphocyte transformation in gonorrhea (7, 11, 17) leave little doubt of the existence of a CMI response in gonococcal infection. However, a number of aspects of the gonococcal CMI response need clarification. Wyle et al. (39) reported a study in which peripheral blood lymphocyte (PBL) transformation was stimulated by both gonococcal and meningococcal antigens in men and women with uncomplicated gonorrhea. The blastogenic responses of PBLs from these individuals were substantially higher than those of normal controls. This demonstrates cross-reactivity between *N. gonorrhoeae* and *N. meningitidis*. The extent of the blastogenic response in women was much greater than in men. Partial purification of these antigens by gel chromatography resulted in reduced cross-reactive responses to the semipurified meningococcal antigen. Female patients demonstrated marked stimulation with the purified gonococcal antigen, whereas male patients showed slight stimulation with purified gonococcal antigen. Therefore, these authors

speculated that CMI may act to limit the spread of gonococcal infection beyond the genital mucous membranes.

Assays which are thought to be in vitro correlates of delayed-type hypersensitivity reactions (lymphocyte activation and production of migration inhibition factor) have been used in a variety of studies of human responses to an array of gonococcal products. Kraus et al. (17), in an attempt to determine whether cell-mediated hypersensitivity develops during a naturally acquired gonococcal infection, cultured lymphocytes from infected individuals with a crude sonic extract of gonococci. The lymphocyte response was quantitated by uptake of labeled deoxyribonucleic acid precursors. Lymphocyte transformation induced by gonococcal antigens occurred in PBLs from some men with gonococcal urethritis. Although transformation was not always demonstrable during the initial infection, it was usually present in patients with two or more episodes of infection. The degree of transformation of the lymphocytes from infected patients with the first episode of gonorrhea did not differ significantly from that observed in noninfected patients. It was suggested that the interval between infection and treatment may have been too short for the patients to develop a significant degree of hypersensitivity. In cultures that were positive for gonococcus-induced proliferation, the maximal stimulation occurred after 5 to 6 days of culture. Esquenazi and Streitfeld (7) confirmed that lymphocyte transformation to gonococcal and to *N. catarrhalis* (now *Branhamella catarrhalis*) sonic extracts could be demonstrated. Lymphocytes from infected patients manifested significant uptake of tritiated thymidine in response to one or more antigens. At 5 weeks posttherapy, lymphocytes were relatively nonreactive to gonococcal antigenic stimulation. Blastogenesis in response to *B. catarrhalis* antigens was also seen in a few gonorrhea patients and in normal controls. Cross-reactivity of *B. catarrhalis* and *N. gonorrhoeae* antigens was indicated by the disappearance of reactivity to the *B. catarrhalis* sonic extract by the lymphocytes of almost all convalescent gonorrhea patients. Grimble and McIllmurray (11), using a crude gonococcal antigen, found positive lymphocyte stimulation in about 85% of patients with gonorrhea. However, using antigens prepared from *N. meningitidis*, they were unable to demonstrate a cross-reactive response in individuals with gonococcal infection. Although these reports clearly indicate that a CMI response occurs in gonococcal infection, this response cannot be correlated with protection from gonococcal infection. In a series of studies of Swedish individuals infected with gonorrhea, Rosenthal and Sandstrom (28–30) found no demonstrable differences in the lymphocyte response to gonococcal antigen in 42 patients with gonococcal urethritis and 18 uninfected controls. One explanation for these results could be the presence of asymptomatic male and female carriers of gonococcal infection in the control group. A second explanation is that the systemic response, as measured by the activity of PBLs, does not reflect what is happening locally. A significant difference in lymphocyte reactivity was noted only between female patients and controls.

ADCC and NK Cell Antigonococcal Activity

Floyd-Keising et al. (8) demonstrated that human PBLs can participate in ADCC against *N. gonorrhoeae*. Acute-phase serum samples from individuals with histories of multiple uncomplicated gonococcal infection, in cooperation with human PBLs, are capable of killing various gonococcal isolates in the absence of complement. Both homologous and heterologous isolates are susceptible to ADCC-mediated antigonococcal activity, with heterologous pelvic inflammatory disease isolates being the most susceptible. In addition to ADCC, unseparated mononuclear cells were capable of natural antigonococcal activity. When the nonimmune mononuclear cells were purified and assayed for their antigonococcal activity, it was demonstrated that adherent monocytes were also capable of a natural antigonococcal activity in the absence of antibody. Inhibition of monocyte phagocytosis by cytochalasin B abolished the natural antigonococcal activity but did not significantly reduce the ADCC-mediated antigonococcal activity. Nonadherent cell populations expressing surface antigens for B, T, or NK cells were not capable of ADCC-mediated antigonococcal activity. However, nonadherent cell populations with either T or NK cell surface markers expressed natural antigonococcal activity. These results indicate that both natural and ADCC-mediated cytotoxicity can be effective against gonococci.

Lymphoid cells of the human fallopian tube have been characterized for cell surface markers and for their participation in ADCC-mediated and natural cytotoxicity. Cooper et al. (3, 4), using discontinuous Percoll gradients to separate fallopian tube lymphoid cells, demonstrated the presence of cells expressing B, T, and NK cell surface markers. The ratio of B to T cells was approximately 1:4. These ratios are consistent with other musocal sites. Lymphoid-cell populations from the human fallopian tubes were purified over Percoll gradients and used as effector cells against gonococci. A cell fraction (ρ = 1.076) from these gradients contained lymphoid cells which were capable of both ADCC-mediated and natural cytotoxicity. PBLs were purified by using plastic adherence and B- and T-cell panning with monoclonal antibodies; these cells were also used as effector cells against gonococci. Populations of T cells contaminated with NK cells were effective in ADCC assays and in natural cytotoxicity. Cytotoxic/suppressor cells ($CD3^+$, $CD8^+$) of high purity were able to express natural cytotoxicity but not ADCC.

Moticka et al. (E. J. Moticka, K. Elliott, T. Hindman, and M. D. Cooper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988; E51, p. 117) described a suppression of phytohemagglutinin-induced mitogenesis by human fallopian tube lymphoid cells (FTLs). Under a variety of experimental conditions, FTLS responded minimally or not at all to several mitogens including phytohemagglutinin, concanavalin A, pokeweed mitogen, and staphylococcal protein A. Studies were performed to determine whether this lack of mitogenic responsiveness was due to the activity of suppressor cells. FTLs were mixed with allogenic PBLs and cultured with the T-cell mitogen phytohemagglutinin under conditions optimal for the stimulation of PBLs. Culturing the PBLs and FTLs in 1:1 or 2:1 ratios depressed the incorporation of [^{3}H]thymidine by more than 50%. As few as 10% FTLs in cultures of PBLs produced consistent suppression of phytohemagglutinin-induced mitogenesis. Control cultures with similar numbers of allogenic PBLs demonstrated no such suppression, indicating that the effect was not due to allogenic interactions. The suppression was attributable to a soluble factor which could interfere with the reactivity of PBLs. The possibility that suppressor cells and suppressor factors play a role in down-regulating antigonococcal cytotoxic T cells is currently under investigation.

CELLULAR RESPONSES IN ANIMALS IMMUNIZED WITH *N. GONORRHOEAE*

Kannungo and Agarwal (15), using a guinea pig subcutaneous chamber model, studied the development of cellular immunity by using lymphocyte and macrophage migration as measurements of CMI. They infected the subcutaneous chambers of guinea pigs with gonococci. At 1 week postinfection there were indications of CMI, which then persisted for up to 7 weeks. The CMI response was detected by using a crude soluble cellular antigen, crude lipopolysaccharide (LPS), and a ribonucleic acid protein antigen prepared from a local clinical isolate. Koostra et al. (16) studied the CMI of guinea pigs immunized with either gonococcal or meningococcal ribosomes and demonstrated a quantitative but not a qualitative specificity. They found about a fivefold difference in the concentration of *N. gonorrhoeae* and *N. meningitidis* skin test antigens necessary to obtain a positive response to guinea pigs immunized with gonococcal ribosomes in complete Freund adjuvant (14). Gonococcal ribosomal ribonucleic acid, ribosomal protein, cell walls, and pili required higher concentrations to obtain a positive skin test than did intact ribosomes. Kwapinski and Chong (18) used purified gonococcal antigens to elicit a CMI in rabbits. A nontoxic protein called B(−/+) was isolated from the cytoplasm of type 1 gonococci. B (−/+) was used to immunize rabbits prior to challenge with live gonococci in the anterior chamber of the eye. The corneas became cloudy within 24 to 48 h, and this was followed by a profuse discharge from the conjunctiva. By day 4 or 5 postinfection, the corneas were clear. In addition, the gonococci thrived when anti-B (−/+) antiserum was injected along with live gonococci into the anterior chamber of the eye.

HOST CELLULAR DEFENSES IN MENINGOCOCCAL INFECTION

Immunity to meningococcal infection is thought to depend primarily on the presence of bactericidal antibody. The production of this protective antibody follows natural infection (9) and vaccination with meningococcal polysaccharide antigens. Subjects who do not possess bactericidal antibody are at risk of developing meningococcal disease (6). Little attention has been paid to the possible protective role of CMI in this infection because of the evidence suggesting a dominant role for antibody in protection against meningococcal disease.

Meningococcal CMI Response

Greenwood et al. (10) studied the CMI response in patients with group A meningococcal meningitis and in normal subjects given group A meningococcal vaccine. They found that lymphocyte responsiveness to both phytohemagglutinin and meningococcal antigens was markedly depressed in patients with acute meningococcal infections. This defect was present whether lymphocytes were cultured in autologous or fetal calf serum. Patients also showed a transient increase in the degree of inhibition produced by group A meningococci in leukocyte migration assays. Meningococci of other groups produced a similar degree of inhibition. Vaccination with group A meningococcal polysaccharide vaccine had no effect on lymphocyte responsiveness to meningococcal antigens or on the inhibitory effect of group A meningococci on leukocyte migration. These negative results must be interpreted carefully, since patients with acute meningococcal meningitis had positive leukocyte inhibition test results which were greater than those seen in recovered patients. Thus, there appears to be a difference in the level of responsiveness depending on the degree of antigenic stimulation.

ADCC and NK Cell Activity against Meningococci

Lowell et al. (20) reported that heat-inactivated serum samples from adults immunized with group C meningococcal polysaccharide vaccine, in cooperation with normal human peripheral blood mononuclear cells, could significantly decrease the viability of group C meningococci. Of the mononuclear-cell populations, K lymphocytes (null cells) and monocytes, but not T or B lymphocytes, were capable of effecting ADCC antimeningococcal activity in this system. The degree of killing in this system was dependent on the incubation time of the reactants, the concentration of the effector cells, and the amount of antiserum used in the assay. When specific antimeningococcal antibodies were absorbed from the serum, ADCC activity was abolished. ADCC antimeningococcal activity was also temperature dependent and could be abolished either by performing the assay at 4°C or by heating the effector cells to 46°C for 15 min prior to the start of the assay. Their data suggest that K cells may play a role in the host immune defense against certain bacterial pathogens. Smith and Lowell (33) reported that ADCC antimeningococcal activity was inhibited when human immune serum was preincubated with the polysaccharide of the immunizing vaccine. Furthermore, the activity against group A meningococci that was induced by heat-inactivated human immune serum was also inhibited by preincubation with that polysaccharide. Neither heterologous polysaccharide nor homologous protein or LPS meningococcal antigens inhibited the activity of either of these sera. Antibodies responsible for ADCC activity against the group C meningococci are also directed against the polysaccharide. The results of these inhibition experiments are, therefore, consistent with those of Roberts (27), who found that opsonophagocytosis of group C and A meningococci by neutrophils in cooperation with sera taken after immunization with group C and group A meningococcal polysaccharides, respectively, was totally inhibited by the homologous polysaccharide. Owing to natural exposure and carriage, most sera of adult humans contain antibodies to both capsular and subcapsular (protein and LPS) meningococcal antigens (9, 40). However, in contrast to the inhibitory action of the polysaccharide, homologous protein and LPS antigens were not inhibitory in this system. These data should not be interpreted as indicating that antibodies to protein and LPS antigens are incapable of inducing ADCC, since blocking the action of antibodies to protein or LPS would still leave antibodies to polysaccharide functionally available in the sera. The data do support the conclusion that if the antibodies to subcapsular LPS or protein are effective in ADCC, they must be of low titer, since blocking of antibodies to polysaccharide did not reveal antibacterial activity. This implies that, since ADCC activity occurs in the absence of added complement, this mechanism may be important to host immune defenses in areas where complement components are relatively low or nonfunctional, such as secretory surfaces including the nasopharynx and the respiratory, intestinal, and urogenital mucosa.

To compare the efficacies of the various immunoglobulin isotypes in ADCC against meningococci, Lowell et al. (22) used purified immunoglobulins from serum samples from

individuals immunized with group C polysaccharide and compared them with immunoglobulins purified from patients convalescing from disseminated meningococcal disease. They found that although IgA is nonbactericidal in the presence of complement, it can induce a cell-mediated antibacterial activity as effectively as IgG can. The amount of IgG required to induce cell-mediated antibacterial activity is similar to the amount required for complement-mediated killing. They further concluded that the amount of either postimmunization or convalescent-phase IgM required to induce complement-mediated killing is 16- to 20-fold smaller than the amount of IgG required. IgM is inferior to IgG in its ability to induce ADCC. In the cell-mediated system, postimmunization IgM is ineffective, and the amount of convalescent-phase IgM required for minimal activity is eight times the amount of convalescent-phase IgG required. Furthermore, the maximal antibacterial index induced by convalescent-phase IgM is 50% less than that which can be induced by IgG. These data suggest that IgG and IgA play a greater role than IgM in the ADCC directed against meningococci in host defense. Lowell et al. further reported (21) that IgA purified from serum samples of patients convalescing from disseminated group C meningococcal disease induced human monocyte-mediated ADCC in the absence of complement. The convalescent-phase IgA was directed specifically against the polysaccharide capsule, and the effective level for ADCC was less than 1 ng of polysaccharide antibody. ADCC activity again was dependent upon the length and the temperature of the test incubation and on the concentration of the monocytes.

CELLULAR RESPONSES IN ANIMALS IMMUNIZED WITH *N. MENINGITIDIS*

A number of aspects of the immune responsiveness of meningococcal infection are still elusive. CMI studies may provide a means of completing the assessment of these responses. There is a demonstrable cellular component to meningococcal immunity which, although antibody dependent, is mediated by cells and is complement independent. The few examples of specific CMI reactions to *N. meningitidis* are limited to migration inhibition in guinea pigs and humans (10, 26, 34, 35) and delayed-type hypersensitivity in guinea pigs (26). Pribnow et al. (26) sensitized guinea pigs to *N. meningitidis* group A by subcutaneous injection of viable meningococci. These animals were skin tested with heat-killed *N. meningitidis* cells, as cell wall preparation of meningococci, and a soluble somatic antigen prepared from the homologous organism. Control skin test substrates included heat-killed *N. gonorrhoeae* cells, purified protein derivative, and Hanks balanced salt solution. Positive 24-h skin reactions, characterized by induration that measured more than 25 mm^2, were produced only by heat-killed meningococci and with the cell wall preparations. The soluble somatic antigen produced only erythema. The meningococci also caused inhibition of migration of macrophages when peritoneal cells from the sensitized guinea pigs were used in the capillary tube MIF test. No inhibition of migration was produced with the control antigens. The delayed-type hypersensitivity reactivity was transferable with viable lymph node cells from the sensitized guinea pigs, but not with dead lymph node cells or with serum.

Sparkes (36) described a preparation of meningococcal antigens extracted in $CaCl_2$ which contained mostly outer membrane proteins and was strongly mitogenic for normal murine B lymphocytes. These meningococcal antigens markedly impaired the in vivo T-cell responsiveness of murine splenocytes. Suppression of the normal splenic T cells occurred with both adherent and nonadherent splenocytes from meningococcal antigen-sensitized mice. B cells were much less affected by the suppression induced by the meningococcal antigens, and only adherent cells could convey in vitro the low-level impairment of B-cell proliferation. Strong T-cell suppression associated with a B-cell mitogen was also produced by *Mycobacterium bovis* BCG and *Corynebacterium parvum*. In another report (34), Sparkes showed that meningococcal antigens had adjuvant activity when administered to mice at the same time as a T-dependent antigen (sheep erythrocytes, [SE]), by increasing the splenocyte plaque forming response in a dose-related manner. However, when SE were given 1 day after meningococcal antigen injection, the subsequent plaque formation was diminished. This decrease was proportional to the dose of meningococcal antigen injected. Splenocytes taken from mice up to 5 days after meningococcal antigen injection actively inhibited plaque formation when mixed with splenocytes immunized with SE 4 days earlier. At 2 days after meningococcal antigen injection, the nonspecific inhibition of plaque formation was due mainly to adherent spleen cells, whereas at 5 days, nonadherent cells had acquired the inhibitory activity. It appears that the degree of activation of adherent cells by meningococcal antigen modulates the subsequent development and secretion of anti-SE antibody-forming cells.

Micusan et al. (24) used an extract from group Y meningococci known to contain protein antigens common to other meningococci to determine the immune response in mice to meningococci. Using delayed-type hypersensitivity as a measure of cell-mediated responsiveness, they could not detect any CMI. Melancon-Kaplan et al. (23), using spleen cells from mice infected with meningococci, demonstrated depressed in vitro plaque-forming cells responses to T-dependent (SE) and T-independent (trinitrophenol [TNP] LPS and TNP-Ficoll) antigens. The inhibition was observed over a wide range of antigen concentrations. The decreased responsiveness of splenocytes from infected mice was due to a selective impairment of B-cell function. Helper-T-cell activity was intact in infected mice, as shown by the ability of T-enriched lymphocytes to cooperate with normal B-enriched lymphocytes in the generation of an anti-SE response. Accessory macrophage function was preserved, since adherent spleen cells from mice inoculated with bacteria were shown to produce normal or increased levels of interleukin-1 and were able to cooperate with normal nonadherent spleen cells in the generation of plaque-forming cell responses against SE. Addition of peritoneal cells from normal animals or extraneous interleukin-1 both failed to restore normal plaque-forming cell responses in cultures of splenocytes from infected mice. B-enriched lymphocytes from infected mice produced poor anti-SE responses when cultured with either concanavalin A supernatant or T-enriched lymphocytes from normal or infected mice. Therefore, the immunological unresponsiveness associated with a meningococcal infection was attributed to a meningococcus-induced defect(s) in B-cell function. In vivo polyclonal B-cell activation leading to clonal exhaustion does not play a major role in the depression of humoral responses, since meningococcal infection induces little or no polyclonal immunoglobulin secretion.

CONCLUSIONS

During the last several decades, there have been sporadic reports concerning the presence of CMI in response to infection with *Neisseria* spp. This includes reports both on the classical manifestations of CMI, such as delayed-type hypersensitivity skin testing, in vitro lymphocyte transformation, and production of soluble lymphokines, as well as more contemporary studies on the activity of cells involved in ADCC and NK cell activity. These reports have been overshadowed by the large number of studies suggesting that immunity to these bacteria is antibody mediated through either complement activation or opsonization. There are several observations which argue against the simplistic notion that only humoral immunity is involved in protecting an individual against infection. These are as follows. (i) Most of the studies of the role of antibody in these infections have been done with immune serum. However, both gonococci and meningococci produce local infections, where serum antibody may not be present. (ii) Complement is deficient or present in only low concentrations in areas where these two species initiate their infections. Furthermore, the predominant immunoglobulin present in the genitourinary tract (for gonococci) and the nasopharynx (for meningococci) is of an isotype (IgA) that does not fix complement. (iii) Antibodies specific for gonococci can be isolated from serum samples of individuals with histories of multiple uncomplicated gonococcal infections. The fact that these individuals develop multiple infections while this antibody is present argues against the postulate that this antibody is protective. Early work on CMI in these infections demonstrated no consistent correlation between the development of specifically sensitized cells and protection. More recent investigations of the ability of human lymphocytes to participate in NK-cell and ADCC activities against gonococci and meningococci suggest that we need to reevaluate the conclusions on the identity of protective immune mechanisms to these two bacteria. This is particularly important as efforts are undertaken to develop new, more effective vaccines for the diseases caused by these organisms.

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Physiology and Metabolism of *Neisseria gonorrhoeae* and *Neisseria meningitidis*: Implications for Pathogenesis

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Gonococci and meningococci are obligate human pathogens that can infect diverse sites within the human host. Each of these sites represents a unique niche with respect to nutrients, environmental factors, and competing microorganisms. The growth environment has a marked effect on the metabolism and cellular composition of *Neisseria gonorrhoeae* and *N. meningitidis*. Altered cellular composition is often reflected by changes in the cell surface that can ultimately affect the interaction of these microorganisms with the human host. The physiology and metabolism of the pathogenic *Neisseria* spp. have not been reviewed since 1979 (41). This review will discuss selected areas that have implications for the pathogenesis of these important microorganisms.

IRON METABOLISM

After entry into the human host, *N. gonorrhoeae* and *N. meningitidis* must multiply to colonize mucosal surfaces and to establish an infection. Cell growth and multiplication require essential nutrients such as iron. To obtain iron, the pathogenic *Neisseria* spp. must acquire it from the host. Despite the relative abundance of iron in the host, there is little free iron because of its sequestration by the iron-binding proteins transferrin (TF) and lactoferrin (LF) (19). In serum and interstitial fluid, iron is associated with TF; in breast milk, semen, and mucosal surfaces, it is associated primarily with LF. Consequently, gonococci and meningococci must possess mechanisms for utilizing the iron associated with the host iron-binding proteins as well as other potential in vivo iron sources such as heme and hemoglobin.

Several studies have implicated iron in the virulence of the pathogenic *Neisseria* spp. Calver et al. (7) demonstrated that injection of ferrous sulfate prior to or injection of either iron sorbitol citrate or iron-dextran concomitantly with injection of *N. meningitidis* increased the lethality of several different meningococcal serogroups for mice by up to a 10^6-fold. The effect of the added iron was partially abrogated by the prior incubation of *N. meningitidis* with Desferal (CIBA-GEIGY Corp.), an iron chelator from which meningococci and gonococci are unable to remove iron (36). Holbein et al. (24) showed that the 50% lethal dose of *N. meningitidis* strains in a mouse model was decreased 10^9-fold by the concomitant administration of iron-dextran with the inoculum. Payne and Finkelstein (50) found that the intravenous inoculation of iron-containing compounds together with avirulent (nonpiliated) gonococci increased the lethality of these avirulent organisms for chicken embryos.

Hafiz et al. (20) observed that high concentrations of ferric citrate appeared to stimulate the reversion of nonpiliated gonococci to piliated gonococci during batch culture in a liquid medium. Odugbemi and Hafiz (49) further demonstrated that the apparent rate of reversion from the nonpiliated state to the piliated state was influenced by both iron and iron chelators. These results are somewhat at odds with recent findings (26) that iron-limited gonococci remained piliated. Nevertheless, these studies provide evidence that iron has an important role in the virulence of the pathogenic *Neisseria* spp. Iron probably influences cellular function(s) since ferric chloride, ferric nitrate, and ferric ammonium citrate did not increase the attachment of gonococci to human spermatozoa (25).

All strains of *N. gonorrhoeae* and *N. meningitidis* are able to grow with 25% iron-saturated TF as their sole source of iron (37). Archibald and DeVoe (1, 2) found that meningococci were capable of obtaining iron from a variety of iron-containing compounds including gastric mucin, ferric citrate, hemoglobin, myoglobin, and human TF. Iron complexed with a number of metabolic organic acids, polyphosphates, and several synthetic polycarboxylic acids was also readily utilized by all meningococcal strains examined. However, *N. meningitidis* was unable to use iron bound to some common hydroxymate- and catechol-type siderophores.

All strains of *N. meningitidis* used LF as an iron source, whereas approximately 60% of the gonococcal strains examined in one study (36) were able to utilize this iron source. The percentage of gonococcal strains that were capable of utilizing LF-bound iron was related to the auxotype of the strain; 86% of prototrophic gonococci were able to utilize LF-bound iron, whereas only 14% of Arg^- Hyx^- Ura^- strains were able to utilize this iron source. Their inability to utilize LF-bound iron has been offered as an explanation for the association of these strains with asymptomatic infection (5). The actual LF concentration in human vaginal mucus varies during the menstrual cycle and is lowest just before menses (13). The implication of this observation in the pathogenesis of pelvic inflammatory disease or disseminated gonococcal infection remains to be determined.

N. meningitidis was shown to acquire TF-bound iron by an iron-repressible, energy-dependent mechanism that required a functional respiratory chain (60). The uptake of iron from TF required direct contact of the TF with the meningococcal surface; during this process, the TF remained extracellular (61). Approximately 70% of the ^{55}Fe taken up by cyanide-treated meningococci was located in the outer membrane in association with a major outer membrane protein with an apparent molecular mass of 36,500 daltons. McKenna et al. (35) have shown that, like meningococci, gonococci remove iron from TF and LF by an iron-repressible, energy-dependent mechanism. The acquisition of TF- and LF-bound iron required direct contact of the protein with the gonococcal cell surface. Gonococci were extremely efficient at utilizing the TF- or LF-bound iron as the sole source of iron for growth; a level of 5% iron saturation was sufficient to support normal growth in vitro, provided that

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enough protein was available to supply the required amount of iron (35).

The mechanism by which gonococci release the iron from TF and LF is unclear. West et al. (63) postulated that the TF and LF bound nonspecifically to the gonococcal cell surface and that the iron was subsequently released by a mechanism involving polyphosphate. The Fe^{3+} bound to TF or LF may also be released by reduction to Fe^{2+}; both cytoplasmic and membrane-bound ferric reductase activities have been detected in gonococci and meningococci (A. E. LeFaou and S. A. Morse, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D20, p. 75). Meningococcal and gonococcal iron-regulated proteins may play a role in the binding of TF or LF, the release of iron, and its transport across the outer and inner membranes. A putative meningococcal TF receptor was recently detected in an assay in which human TF conjugated to horseradish peroxidase was used (54). Further studies involving sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblot analysis showed that it was a 71,000-dalton, iron-regulated outer membrane protein (54). Competitive-binding experiments indicated that the receptor exhibited a specificity for human TF and was distinct from the LF receptor. An LF-binding protein (105,000 daltons) was subsequently identified in three different meningococcal strains by using biotinylated human LF and streptavidin-agarose (55). The LF-binding protein exhibited a specificity for human LF, and its synthesis was regulated by the level of iron in the medium. The specificity exhibited for human TF and LF may be an important factor in the host specificity of these pathogens.

Free hemoglobin or hemoglobin bound to haptoglobin can be used as an iron source by most gonococcal and meningococcal strains (17). *N. gonorrhoeae* and *N. meningitidis* can also utilize free heme as an iron source (37, 66), but not when it is complexed to hemopexin or albumin (17).

Yancey and Finkelstein (67) first reported the production of a siderophore by disseminating strains of *N. gonorrhoeae*. Subsequently, they isolated a dihydroxamate-type siderophore from concentrated culture supernatants of *N. gonorrhoeae* and *N. meningitidis* (66). However, Archibald and DeVoe (2) and Norrod and Williams (48) were unable to detect any siderophore activity in spent culture supernatants from these organisms. These conflicting reports were recently clarified by West and Sparling (64), who were also unable to detect siderophore activity in *N. gonorrhoeae*. They further showed that the quantity of siderophore detected by bioassay of culture supernatants from *N. gonorrhoeae* was never greater than the amount already present in the uninoculated medium.

Gonococci are capable of utilizing siderophores produced by other microorganisms. Yancey and Finkelstein (66) have reported that gonococci can utilize the dihydroxamate siderophores aerobactin, arthrobactin, and schizokinen. West and Sparling (65) confirmed that gonococci could utilize ferri-aerobactin as a sole iron source. In addition, they probed gonococcal genomic deoxyribonucleic acid with the cloned *Escherichia coli* aerobactin-biosynthetic genes (*iucABCD*) as well as the aerobactin receptor (*iutA*) and hydroxamate utilization (*fhuCDB*) genes; hybridization was detected with *fhuB* sequences but not with the other genes. West and Sparling (65) identified and cloned the region of the gonococcal genome that exhibited homology with *fhuB* and demonstrated its ability to complement *fhuB* mutations in *E. coli*.

Norqvist et al. (46) were the first to report that gonococci expressed several iron-regulated proteins when grown under iron-limited conditions. These iron-regulated proteins were heterogeneous with respect to both their relative migration during sodium dodecyl sulfate-polyacrylamide gel electrophoresis and their presence in different strains. Only one protein, with an apparent molecular mass of 97,000 daltons, appeared to be conserved among all the gonococcal strains examined. Mietzner et al. (40) confirmed the previous observation and identified a previously unrecognized iron-regulated protein with an apparent molecular mass of 37,000 daltons. This protein comigrated with the protein I of some strains and was resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis only under conditions of increased ionic strength, which permitted protein I and the 37,000-dalton protein to migrate as distinct bands. Although the expression of most of the other iron-regulated proteins varied among strains, the 37,000-dalton protein was common to all the gonococcal strains examined. Two-dimensional peptide maps of this protein obtained from two unrelated gonococcal strains were identical, suggesting that it was highly conserved.

West and Sparling (64) examined the expression of gonococcal iron-regulated proteins during growth in medium containing different iron sources. They found that, with the exception of the 37,000-dalton protein that was expressed under all conditions of iron limitation, most of the iron-regulated proteins were not coordinately regulated. The expression of the 37,000-dalton protein under all conditions of iron limitation and by all of the strains examined prompted speculation that this protein played a key role in the acquisition of iron by *N. gonorrhoeae*.

The gonococcal 37,000-dalton protein was purified by a combination of selective extraction with cetyltrimethylammonium bromide and column chromatography and used to produce both rabbit monospecific antiserum and murine monoclonal antibodies (39). Using these reagents, Mietzner et al. (38) found that this protein was antigenically conserved among strains of *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, and *N. cinerea*. Two-dimensional peptide maps and N-terminal amino acid sequences from the 37,000-dalton proteins isolated from these species confirmed that the primary structure of the protein was highly conserved (C.-Y. Chen, Ph.D. thesis, Oregon Health Sciences University, Portland, 1988). The antigenic and structural conservation of these 37,000-dalton proteins, particularly among the pathogenic members of the genus *Neisseria*, suggested that they may serve a common function in pathogenesis. Acute-phase serum specimens, as well as vaginal wash fluids from patients with disseminated gonococcal infection and uncomplicated gonococcal infections (18, 44) and with meningococcal infection (18), contained immunoglobulins that reacted with the 37,000-dalton protein, indicating that this protein is both expressed and antigenic in vivo.

The 37,000-dalton protein, purified to homogeneity from both *N. gonorrhoeae* and *N. meningitidis*, contains approximately 1 mol of Fe^{3+} per mol of protein (39, 42, 43). Furthermore, when isolated from gonococci grown in a liquid medium containing [^{55}Fe]TF as the only source of iron, the protein was labeled with ^{55}Fe (42). Whether the ^{55}Fe was directly transferred from TF to the 37,000-dalton protein or whether one or more intermediate steps were involved in this process is not known. The susceptibility of the 37,000-dalton protein to proteolytic cleavage following treatment of intact gonococci with the serine protease lysosomal cathepsin G suggested that certain portions of this protein were surface exposed (56). This surface exposure, subsequently confirmed by surface peptide mapping (C.-Y.

Chen and S. A. Morse, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D-185, p. 102), indicated that a direct interaction between TF and the 37,000-dalton protein was possible.

The gene encoding the gonococcal 37,000-dalton protein has been cloned in three overlapping fragments and sequenced (S. Berish, T. A. Mietzner, and S. A. Morse, unpublished data). The consensus amino acid sequence predicted a mature protein containing 308 amino acids, and the molecular weight based on this sequence was 33,571. Hybridization analysis suggested that there was a single copy of this gene in the gonococcal chromosome.

Tarkka and Sarvas (62) recently reported cloning a 37,000-dalton protein from *N. meningitidis*. However, this cloned gene did not hybridize to several different oligonucleotide probes specific for the 37,000-dalton gonococcal and meningococcal iron-regulated proteins and hence does not appear to be the same protein (C. Genco, unpublished results).

The availability of mutants that are altered in their ability to utilize specific iron sources has enabled researchers to more closely examine the relationship between iron and virulence and to study the mechanism of Fe uptake by *N. gonorrhoeae* and *N. meningitidis*. Streptonigrin, an aminoquinone, causes deoxyribonucleic acid degradation and requires iron for its bactericidal effect (68). It has been used to selectively enrich for mutants altered in their ability to utilize specific iron sources. Dyer et al. (15) described a mutant of *N. meningitidis* that was impaired in its ability to use TF-bound iron. This mutant did not produce the 85,000- and 95,000-dalton iron-repressible outer membrane proteins. However, genetic transformation experiments indicated that these outer membrane proteins were probably not responsible for the TF-deficient phenotype. Recently, Dyer et al. (16) isolated a pleiotropic iron uptake mutant of *N. meningitidis* that lacks a 70,000-dalton iron-regulated protein. This mutant was markedly deficient in the uptake of iron from TF, LF, citrate, and aerobactin. These authors suggested that the 70,000-dalton protein may be involved in the uptake or utilization of these iron sources; however, the data supporting this hypothesis were not conclusive.

We have isolated several mutants of *N. gonorrhoeae* 340 that grew normally with heme as a sole source of iron but were deficient in the uptake of TF-bound iron (our unpublished data). The parental strain was virulent in the mouse subcutaneous chamber model, whereas the mutants were avirulent. Results from these studies indicated that gonococcal strains defective in iron utilization were not capable of sustained growth in vivo and suggested that iron utilization was required to initiate an active infection.

Despite these studies on iron-regulated proteins and iron uptake systems, our knowledge of the biochemistry and mechanism of iron transport by the pathogenic *Neisseria* species is still rudimentary.

SULFUR METABOLISM

In contrast to other bacteria, gonococci and meningococci are restricted in the way they can obtain sulfur for growth. All strains of gonococci and some strains of meningococci require cystine (or cysteine) for growth (8).

DeVoe et al. (14) identified thiosulfate reductase activity in *N. meningitidis*. Other forms of sulfur that supported meningococcal growth included sulfate, sulfite, bisulfite, thiosulfate, dithionite, hydrosulfide, thiocyanate, L-cysteine, L-cystine, reduced glutathione, methionine, mercaptosuccinate, and lathionine (52).

LeFaou (31) demonstrated the presence of thiosulfate sulfur transferase, trithionate reductase, and tetrathionate reductase activities in *N. gonorrhoeae*. Despite a report that gonococci lacked sulfite reductase (32), Norrod (47) observed that the addition of sulfite to medium already containing cysteine and cystine resulted in an alteration in the structure of gonococcal lipooligosaccharide.

AMINO ACID METABOLISM

Auxotyping of gonococci (and to a lesser extent meningococci) has been used to assist epidemiologic investigations. Gonococcal amino acid metabolism has been reviewed previously (41); more recent investigations are described below.

Arginine biosynthesis and utilization are the most extensively studied, no doubt as a result of the predominance of the Arg$^-$ Hyx$^-$ Ura$^-$ auxotype among gonococci isolated from disseminated infections. A high proportion of Arg$^-$ strains utilized ornithine in place of arginine (9), suggesting that there were defects in the conversion of α-*N*-acetylornithine to ornithine or in the carbamylation of ornithine to citrulline. Ornithine transcarbamylase activity was found (9) and subsequently characterized (57) in strains that were incapable of growing on ornithine, suggesting that the inability to utilize ornithine was due to other defects. Powers and Pierson (53) purified and characterized ornithine transcarbamylase from *N. gonorrhoeae* and found that the enzyme was similar to that described in other bacteria.

Shinners and Catlin (58) examined Arg$^-$ Ura$^-$ gonococcal strains for glutamate acetyltransferase, aspartate transcarbamylase, orotate phosphoribosyltransferase, and carbamyl phosphate synthetase. They discovered that strains incapable of growth on ornithine lacked carbamyl phosphate synthetase. The lack of this enzyme resulted in a concomitant requirement for pyrimidines, since carbamyl phosphate is also a precursor in the biosynthesis of these compounds.

A large number of clinical isolates of *N. gonorrhoeae* have been examined and grouped according to their amino acid requirements (23). These six groups included nonrequiring, Pro$^-$, Orn$^-$, Pro$^-$ Cit$^-$ Ura$^-$, Orn$^-$ Ura$^-$ Hyx$^-$, and Cit$^-$ Ura$^-$ Hyx$^-$ isolates. Several nonrequiring and Pro$^-$ auxotrophs were further studied to determine their requirement for proline and the kinetics of growth on proline (21). Growth in defined medium was very sensitive to proline concentration and to whether starch or fatty-acid-free bovine serum albumin was added as a protective agent. Chen and Buchanan (10) purified and characterized a proline iminopeptidase that allowed Pro$^-$ auxotrophs to grow in a medium consisting in part of proline-containing polypeptides. Proline utilization by nonrequiring, Pro$^-$, and thiamine-requiring auxotrophs was recently studied by Pillon et al. (51). They determined that proline was utilized efficiently as an energy source via reactions involving the tricarboxylic acid cycle.

Aromatic amino acid metabolism has been recently investigated. Some strains of gonococci are sensitive to growth inhibition by phenylalanine (22). This phenylalanine sensitivity was found to be due to feedback inhibition of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate, an enzyme active early in the common pathway of phenylalanine and tyrosine biosynthesis (3). Addition of tyrosine to the medium negated the phenylalanine repression.

ANAEROBIC GROWTH

N. gonorrhoeae proliferates and grows in the presence of a milieu of strict anaerobic organisms. Its normal sites of

infection are the cervix, rectum, pharynx, and genitourinary tract. In addition, gonococcal pelvic inflammatory disease is often a mixed infection with obligate anaerobes (6).

Kellogg et al. (29) reported the growth of *N. gonorrhoeae* under low oxygen tension and its survival under anaerobic conditions. Short et al. (59) reported the survival of both clinical and laboratory isolates of *N. gonorrhoeae* under anaerobic conditions. Of the laboratory strains examined, all maintained viability better at 27°C than at 37°C, and the Arg⁻ Hyx⁻ Ura⁻ strains survived better than strains of other auxotypes. Of 21 clinical isolates examined, 3 were able to grow anaerobically on prereduced Martin-Lewis agar plates; attempts to subculture these isolates anaerobically were not successful. The addition of the electron acceptors nitrate or fumarate or both to the medium did not support the anaerobic growth of these strains.

Knapp and Clark (30) examined 204 strains of *N. gonorrhoeae* for their ability to grow anaerobically with nitrite as the terminal electron acceptor. All strains grew anaerobically with subtoxic concentrations of nitrite. The generation time of *N. gonorrhoeae* growing anaerobically in the presence of nitrite was almost as rapid as that of gonococci growing aerobically in the same medium lacking nitrite; the final turbidity that was achieved anaerobically was comparable to that obtained aerobically. Cytochrome oxidase and nitrite reductase were produced constitutively under both anaerobic and aerobic conditions. There was no growth of *N. gonorrhoeae* with sulfite as a terminal electron acceptor. The ability of gonococci to grow either aerobically or anaerobically may allow them to proliferate on any mucosal surface of the body to which they can attach and may explain how they can be isolated from mixed infections with obligate anaerobes.

Clark et al. (11) compared the outer membrane protein composition of aerobically and anaerobically grown *N. gonorrhoeae* strains by one- and two-dimensional polyacrylamide gel electrophoresis. Anaerobic growth of *N. gonorrhoeae* resulted in both the induction and the repression of outer membrane proteins. The expression of at least three proteins (Pan 1 to Pan 3) was increased during anaerobic growth. Pan 1 and Pan 2 were highly conserved among gonococcal strains in that they had identical apparent molecular masses. During continuous culture, a protein with a molecular mass similar to that of Pan 1 was observed only under anaerobic conditions, whereas other proteins that were expressed under these conditions were also observed under other conditions of nutrient limitation (26). Thus, Pan 1 may be specifically induced by anaerobiosis, and Pan 2 and Pan 3 may be synthesized in response to nutritional stress. In addition, Clark et al. (11) observed at least five other proteins (Pox 1 to Pox 5) that were expressed at higher levels in aerobically grown cells. Anaerobic growth did not significantly alter the expression of other major outer membrane proteins (proteins I, II, III, pilin, and H.8). No apparent differences in lipopolysaccharide composition were observed between aerobically and anaerobically grown gonococci.

The expression of new outer membrane proteins during anaerobic growth indicated that these proteins were under genetic regulation and thus may be involved in colonization or pathogenesis at anaerobic sites. Indeed, Clark et al. (12) have found that serum specimens from convalescent patients with pelvic inflammatory disease or uncomplicated gonococcal infection, but not normal human serum, contained immunoglobulins that strongly reacted with Pan 1 in Western blots.

CONTINUOUS CULTURE

Studies of the pathogenesis of *N. gonorrhoeae* and *N. meningitidis* have classically involved the use of bacteria grown either on solid medium or in liquid culture to log phase. Although they provide important data, these studies suffer from problems that are inherent with the use of plate- or batch-grown cells. Bacterial growing in a closed system (e.g., batch culture) are exposed to a continually changing environment. In contrast, the in vivo environment is relatively constant, as ensured by homeostatic mechanisms of the host. The constraints of using bacteria grown in closed systems can be circumvented by using continuous culture. The chemostat is ideal for strictly controlling growth conditions and providing a uniform, constant environment.

Results of continuous-culture studies have demonstrated that nutrient-limited growth can produce significant alterations in the cell envelope. Peptidoglycan obtained from chemostat-grown gonococci had significantly less O-acetylation than that obtained from batch-grown cells did, although no differences in cross-linking were observed (4). Leith and Morse (33) grew nonpiliated gonococci under glucose-limited conditions and observed decreasing quantities of several outer membrane proteins when the dissolved oxygen concentration was reduced from 54 to 4% of saturation. Subsequently, gonococci grown under glucose limitation at high dissolved-oxygen levels (80 to 100%) became completely serum resistant at specific growth rates greater than 0.3 (45). This decrease in serum sensitivity occurred concurrently with decreasing cell surface hydrophobicity and increasing amounts of lipopolysaccharide serotype antigen. Coincidently, total serum resistance occurred at the point at which glucose was no longer totally depleted.

Manchee et al. (34) grew piliated gonococci in continuous culture in a defined medium at a dissolved oxygen concentration of 20%. The bacteria remained piliated for longer than 21 days and were virulent when evaluated in the guinea pig subcutaneous chamber model. Keevil et al. (26, 27) found that iron-limited gonococci remained piliated and were highly virulent for guinea pig subcutaneous chambers. However, switching to glucose-limited growth resulted in loss of piliation but retention of virulence. In contrast, cystine-limited gonococci were piliated but avirulent. Keevil et al. (28) have recently studied plasmid maintenance in two gonococcal strains grown in a chemostat under glucose limitation. One strain retained the conjugative (24.5-megadalton), β-lactamase (3.2-megadalton) and cryptic (2.6-megadalton) plasmids during 96 generations in continuous culture. The other strain, containing the conjugative, cryptic, and 4.4-megadalton β-lactamase plasmids, lost the conjugative plasmid after 30 generations; the 4.4- and 2.6-megadalton plasmids were lost by 100 generations.

ADDITIONAL COMMENTS

Studies of the physiology and metabolism of the pathogenic *Neisseria* species are important in furthering our knowledge of the interaction of these microorganisms with the human host. Studies similar to those cited in this review will complement studies on the genetics, molecular biology, and pathogenesis of *N. gonorrhoeae* and *N. meningitidis*. A thoroughly integrated approach will enable investigators to solve problems associated with the prevention of infections caused by these microorganisms.

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Protein I: Structure, Function, and Genetics

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The study of the major outer membrane (OM) protein, protein I (PI), of *Neisseria gonorrhoeae* has proven to be as fascinating, and as frustrating, as every other aspect of this remarkable bacterium. However, intensive studies over the last few years have provided significant insight into the structure, function, and genetics of this abundant, surface-exposed porin protein. There are two forms of PI, which share up to 80% homology. The diversity of the exposed portion of the molecules among strains presents an ever-changing immunogenic and antigenic dilemma to the reinfected host. Yet, it is the variability of the PI surface epitopes that has provided a precise method of serotyping *N. gonorrhoeae*, making it possible to monitor the prevalance and spread of strains within the host population and correlate functional differences between strains with PI structural types.

Recent successes in cloning and sequencing of both PI structural subclasses have already contributed to our understanding of the mechanism(s) whereby *N. gonorrhoeae* generates PI structural diversity. Continued investigation is needed to clarify the relationship between PI type and disseminated disease, resistance to serum killing, auxotypic requirements, interaction with host cells, and resistance to antibiotics. Observations regarding the ability of PI to interact with eucaryotic membranes suggest a role for PI in the pathogenesis of *N. gonorrhoeae*, whereas immune responsiveness of patients to PI points to the possibility of a PI vaccine. Clearly, at the molecular level, PI represents all the mystery and intrigue that *N. gonorrhoeae* presents at the organism level. Certainly, much remains to be learned before we have a complete picture of the structure, function, and genetics of PI.

STRUCTURE

PI (73) is known to exist in two structurally related forms, designated subclass PIA and subclass PIB (61), which have different orientations in the OM (2, 4). A given strain or *N. gonorrhoeae* expresses a single, invariant PI of one of the other subclass, which accounts for up to 60% of the protein in the OM (36), whether grown aerobically or anaerobically (16). There is significant structural variation within the subclasses, resulting in proteins of different apparent molecular mass (52, 72), different isoelectric points (with some as low as 5.5, but most close to 8.0) (3), and unique immunological reactivities (6, 45, 61–63, 75). PIAs tend to be smaller than PIBs, ranging in apparent molecular mass from about 34 to 36.5 kilodaltons (kDa), whereas PIBs range from about 36 to 38 kDa (38, 52, 72, 73) (Fig. 1). Regardless of subclass, PIs appear to associate as trimeric porins which form hydrophilic channels through the OM (3, 19, 51, 78). Proteins of both subclasses show immunological variation (6, 45, 61–63, 75), which tends to be localized in surface-exposed portions of the molecules (13, 40, 42, 44, 46, 75). PIs also interact with other OM components, such as protein III (PIII) (55, 74) and lipooligosaccharide (LOS) (31), to form complex OM structures.

The amino acid sequences of a representative PIA (strain FA19) (14) and two PIBs (strains R10 [23] and MS11 [13]) are known. PIA and PIB have identical 19-amino-acid leader sequences which are cleaved to produce the functional protein (13, 14, 23). There is 65 to 80% homology between PIA and amino acid sequences of the two PIBs, confirming similarities previously observed by peptide mapping (2, 39, 40, 61, 72). Peptide mapping studies of PIAs and PIBs and sequence data for two PIBs (13) indicate that there is a higher degree of homology within the subclasses (2, 44, 61, 72). The predicted molecular masses from the deoxyribonucleic acid (DNA) sequences are about 34 kDa for the PIA and 35.5 to 36 kDa for the PIBs. These masses are remarkably close to the apparent molecular masses derived by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and are consistent with the masses of *Escherichia coli* porin proteins (14, 20, 23). Additionally, the PI sequences reveal an absence of long hydrophobic stretches and a lack of cysteine, and they have about 23% sequence homology with *E. coli* trimeric porins (23). Hydropathy patterns of the predicted PI sequences are also consistent with patterns of *E. coli* OmpC and OmpF porins (14), providing evidence that PI molecules act as porin proteins, forming hydrophilic channels across the OM (3, 19, 51, 78).

Sequence differences between the PIA and PIB subclasses result in different orientations of the PIs in the OM (Fig. 2). PIAs are oriented in the OM in such a manner that they have a relatively small portion (15 to 20 amino acids) of the protein exposed on the bacterial surface (2, 4, 42, 44, 68). PIBs traverse the membrane at least twice, having, perhaps, both termini embedded in the OM (2, 4, 68). Thus, PIAs are resistant to in situ proteolysis by trypsin and α-chymotrypsin (2, 4, 44, 68), with only a short portion of the molecule being susceptible to cleavage by proteinase K. Cleavage of purified PIAs from two strains of *N. gonorrhoeae* with leucine aminopeptidase, followed by immunoblot analysis, indicated that the N-terminus is the exposed portion of PIAs (42). A very recent study (13), in which shuttle mutagenesis was used to introduce a selectable marker near the PI gene, used transformation to locate surface epitopes of PIA and PIB in PIA-PIB hybrids. This study confirmed the N-terminal exposure of PIA, but suggested that there may be C-terminal exposure as well.

PIBs are very susceptible to in situ cleavage by exogenous proteases such as trypsin, α-chymotrypsin, and proteinase K and by neutrophil proteases such as elastase and cathepsin G (W. M. Shafer, personal communication), which cleave a central portion of the molecule, leaving two membrane-bound fragments (2, 4, 44). Cleavage of purified PIBs with CNBr (76) and endopeptidases (21), followed by immunoblot analysis, demonstrated that the central portion of PIB is the surface-exposed portion of the molecule. Analysis of PIA-PIB hybrids suggested that a portion of the N terminus may also be exposed in PIBs (13). The portions of the PIs that are exposed on the surface tend to be variable (13, 40, 42, 44, 46, 75) and possess epitopes which allow for immunological classification of strains based on the structural variation within the PIA and PIB subclasses (6, 46, 61–63, 75).

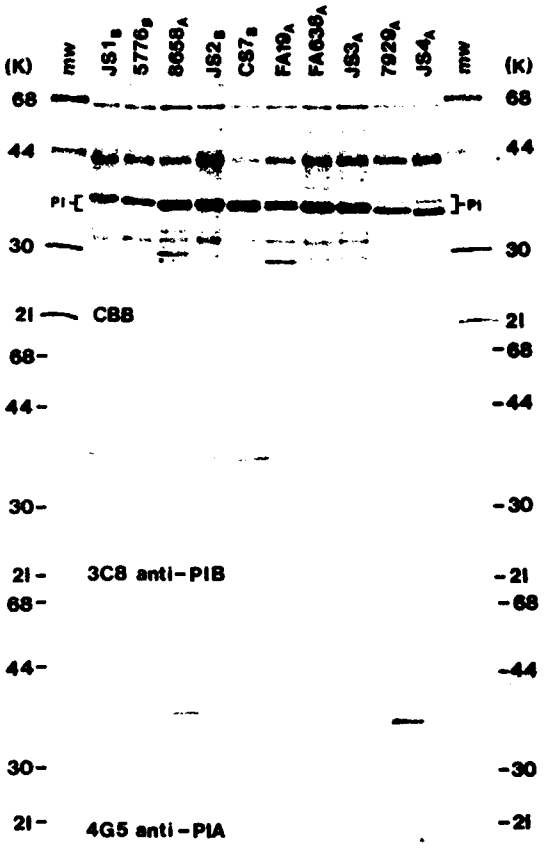

FIG. 1. Separation of whole-cell lysates of *N. gonorrhoeae* JS1, 5776, 8658, JS2, CS7, FA19, FA638, JS3, 7929, and JS4 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 12.5% gel. The top panel is the central portion of a Coomassie brilliant blue (CBB)-stained gel which shows the range of apparent molecular masses of PI. The apparent molecular masses, expressed in kilodaltons (K), of the PI of each strain, as determined by comparison with the Bio-Rad low-molecular-weight markers (mw) are as follows: JS1, 37.7 kDa; 5776, 36.9 kDa; 8658, 37.3 kDa; JS2, 36.3 kDa; CS7, 35.7 kDa; FA19, 35.7 kDa; FA638, 35.7 kDa; JS3 35.7 kDa; 7929, 35.2 kDa; and JS4, 34.6 kDa. The middle panel is an immunoblot using the PIB-specific 3C8 MAb (75), and the bottom panel is an immunoblot using the PIA-specific 4G5 MAb (75). Both blots were probed with anti-mouse IgG–horseradish peroxidase. The PI subclass of each strain, as determined by ^{125}I-labeled peptide mapping (42), is given as a subscript to each strain designation. Note that although the PIBs tend to be larger than the PIAs, there is considerable overlap in their apparent molecular masses. Also note that not all PIs bind these widely reactive anti-PI MAbs (e.g., strains JS3 and JS4), necessitating further analyses to determine the PI subclass of these nonreactive strains.

Initial efforts to serotype *N. gonorrhoeae* involved the use of antisera raised against OM vesicles (36). PI was a dominant antigen in these vesicles (36, 52), indicating that it might be useful in serotype analyses. Sandstrom and Danielsson (63), using absorbed polyclonal antisera in coagglutination assays, were able to separate *N. gonorrhoeae* into three serogroups designated WI, WII, and WIII. A second method of serogrouping, involving the use of anti-PI antibodies in an enzyme-linked immunosorbent assay, was developed by Buchanan and Hildebrandt (6). This system divided gonococci into nine serotypes. Further studies showed that both serogrouping systems correlated with the PI subclass: the WI strains and serogroup 1 to 3 have a PIA, and the WII and WIII strains and serogroups 4 to 9 have a PIB (61, 63). A series of PI-specific monoclonal antibodies (MAbs) have since been produced (Fig. 1) which react with different epitopes on or near the surface-exposed portion of the PI molecules (46, 75). On the basis of the reactivity patterns to these "banks" of PIA- or PIB-specific MAbs in coagglutination assays, systems of serotyping have been developed that classify strains into serovariants, or serovars. Thus, strains of *N. gonorrhoeae* can be described by their PI subclass and the reactivity of the PI with the serovar MAbs (e.g., PIA-1 indicates that this strain has a PIA that reacts with all six of the serovar MAbs) (46, 65, 75). The large number of serovar patterns demonstrates the wide structural variation in or around the surface-exposed regions of the PIs (45, 46, 75). Exposure analyses have confirmed the structural variability of the surface-exposed regions of the PIs (21, 40, 42, 44, 76).

Primary structural differences are not the only factors affecting PI structure in the OM. PI molecules interact to form trimeric porin structures (3, 51). PIII also interacts with PI in the OM in a ratio of three PIII molecules to one PI molecule, forming a complex that can be chemically cross-linked (55). Swanson et al. (74) confirmed an in situ association of PI and PIII by demonstrating that anti-PIII MAb coprecipitated PI. PIII may therefore participate in the formation, stabilization, and/or operation of the trimeric PI pore. A noncovalent association between LOS and PI has also been demonstrated (31). Although the nature of this association is not known, variation of LOS structure does correlate with differences in PI exposure in strains of *N. gonorrhoeae* having structurally identical PIAs or PIBs (R. C. Judd and W. M. Shafer, Mol. Microbiol., in press). Recent studies have demonstrated that PI is associated with peptidoglycan (PG) (S. A. Hill, Ph.D. thesis, University of Montana, Missoula, 1987), perhaps serving to anchor the OM to PG at critical sites. Neither protein IIs, pili, nor H.8 antigen appear to interact directly with PI in the OM (32, 60). A recent study by Robinson et al. (60) demonstrated that the availability of PI to bind PI-specific MAb and polyclonal antibody in intact cells varied extensively, even within the same culture. This raises the possibility that structurally identical PIs do not have the same orientation in all cells; that PI is not expressed in all cells; or that some other, as yet undescribed, interaction between PI and another OM component(s) is occurring differently in different cells (60).

FUNCTION

The known function of PI is to form hydrophilic pores across the OM (porin) (3, 19, 51). The porin function of PI is clearly necessary for the survival of the bacterium, allowing nutrients to penetrate into the cell and waste materials to exit. Other possible cellular functions of PI have been suggested, such as translocation into recipient membranes (3, 49) and association with PG (Hill, Ph.D. thesis). PI subclass has been associated with such traits as serum resistance (12, 29, 38, 57), resistance to antibiotics (1, 8, 33, 35, 59), auxotype (5, 24, 47, 54, 64), and expression of type 1 or type 2 immunoglobulin A1 (IgA1) protease (54). The role of PI in pathogenesis remains elusive, but the PI subclass has been associated with certain clinical states such as disseminated gonococcal infection and localized mucosal disease (5, 9, 50, 54, 56, 64).

The porin activity of PIs has been investigated by analyzing their ability to conduct electric currents when placed in lipid bilayers (3, 49, 51, 78). PIs inserted into artificial membrane bilayers conducted current in bursts, indicating

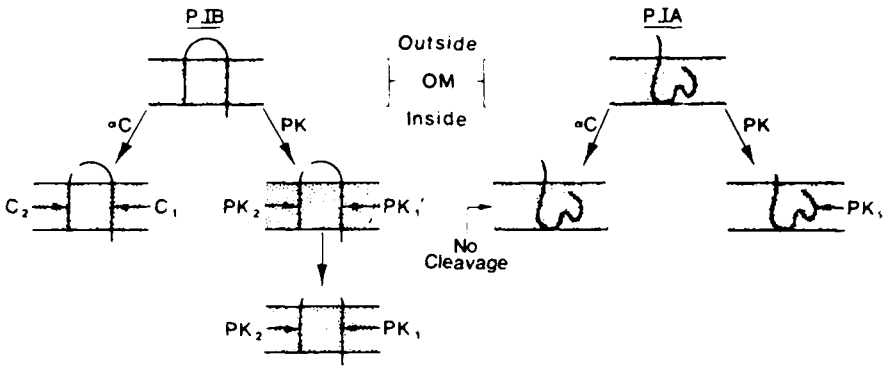

FIG. 2. Model of PIB and PIA in OMs of *N. gonorrhoeae* based on in situ cleavage by α-chymotrypsin (αC) or proteinase K (PK). The PIs were cleaved to yield the fragments shown in this model. C_1, C_2, PK_1', which is further cleaved to produce the PK_1 fragment, and PK_2 are produced when PIB-bearing strains are treated with α-chymotrypsin proteinase K. The PK_1 fragment is generated when PIA-bearing strains are treated with proteinase K. PIAs are resistant to in situ cleavage by α-chymotrypsin and trypsin. On the basis of these cleavage patterns, PIB has a central portion of the molecule exposed on the surface, with both termini embedded in the membrane, whereas PIAs have a short portion of the N terminus (42) exposed on the bacterial surface. Reproduced from *Infection and Immunity* (2) with permission of the publisher.

the pores existed in two states, open or closed (3, 49). Moreover, gonococcal PIs showed incremental voltage gating of current across a lipid bilayer in a manner characteristic of trimeric porins (20, 51). Thus, PIs appear to be porins with three PI molecules combining to form hydrophilic channels, through the OM (Fig. 3). The diameter of the pore has been estimated to be 2.5 nm (19), with an anion-selective channel ranging in Cl^-/K^+ selectivity from 6:1 (for PIBs) to 3:1 (for PIAs) (3, 78).

Further studies with lipid bilayers have indicated that gonococcal PIs spontaneously insert into these membranes in an inverted manner (3, 49). The rate of insertion of *N. gonorrhoeae* PIs was lower for PIBs (5 to 10 pores per h) than for PIAs (100 pores per h) when 10^6 organisms were placed in the test system. In comparison, *Neisseria sicca* did not transfer porin function to the bilayer, even when 10^9 organisms were used, whereas 10^6 organisms of *Neisseria meningitidis* transferred up to 1,500 pores per h (3, 49). Interestingly, gonococcal strains isolated from patients with disseminated disease had greater pore-forming activity than did strains isolated from mucosal sites (3), perhaps reflecting the observation that 80 to 90% of disseminated gonococcal infection isolates express the PIA subclass. It has been demonstrated that PI can be inserted into erythrocytes (3) and that transfer is facilitated when the recipient membrane is more fluid than the bacterial OM (3). The effect of this transfer on pathogenesis is unclear. It has been shown that neutrophils pretreated with purified PI had a decreased ability to exocytose granules and, in response to stimulation with *N*-formyl-Met-Leu-Phe, failed to increase their surface area and did not aggregate as well as control cells (25). PI has also been shown to bind calmodulin (3), a eucaryotic-cell-regulatory molecule. The effect of these activities on pathogenesis remains unclear, but is seems reasonable that PI mediates important events in host cells that contribute to bacterial survival and pathogenesis.

N. gonorrhoeae lacks a protein analogous to Braun's lipoprotein found in *E. coli* (26). Recently, PI has been shown to be one of several PG-associated proteins (30), suggesting that PI may help bind the OM to PG. This is supported by the observation that PI binds lectins that are specific for sugars found in gonococcal PG (Hill, Ph.D. thesis). Moreover, antibody raised against highly purified PIB (R.C. Judd, Abstr. Third Biannual UA/UC Conf. Pathogenic Bacteria, abstr. no. 5, p. 1) cross-reacted extensively with several other PG-associated proteins, while antibody made to a purified 60-kDa PG-associated protein reacts with PI, indicating that PI shares epitopes, probably of PG origin, with these molecules.

Protein I has been associated with several traits that are important to disease. The availability of serotyping reagents (46, 75) has made it possible to correlate PI subclass with many characteristics of the organism as well as the nature of the infection caused by a particular isolate. PIA has been associated with resistance to killing by normal human serum (29, 38, 57). As with most things gonococcal, the association is not absolute. A recent study comparing susceptibility to killing by normal human serum in transformant strains which have identical PIAs or PIBs in differing LOS and H.8 antigen backgrounds demonstrated that PI subclass does not account for serum resistance (R. K. Pettit, J. C. Szuba, and R. C. Judd, unpublished data), confirming that other bacterial components must play a role in resistance (11, 12, 69–71).

There does appear to be a relationship between PI subclass and antibiotic resistance. Several studies indicate that resistance to rifampin (8), thiamphenicol (8), ampicillin (59), and penicillin (59, 67) correlates strongly with the PIB subclass. Correlations between PI subclass and antibiotic resistance are somewhat dependent on geographical location (33, 35, 59), and both PIA- and PIB-bearing strains are capable of plasmid- and chromosome-mediated resistance to antibiotics. There is a consistent correlation between the arginine$^-$ hypoxanthine$^-$ uracil$^-$ (AHU^-) auxotype and PIA (5, 24, 47, 54, 64). Other auxotypes do not correlate as strongly with a particular PI subclass. Therefore, serotyping schemes have been developed which combine the PI serovar and auxotype to classify gonococcal isolates (24, 45).

Another association regarding PI subclass is the production of type 1 IgA1 protease, which is produced predominantly by AHU^-, PIA-bearing strains, and type 2 IgA1 protease, which is produced by many other auxotypes and serovars (54). The AHU^- auxotype and PIA subclass do correlate with the ability to cause disseminated infection (5, 9, 47, 50, 54, 56, 64); the majority (>85%) of blood isolates possess the PIA subclass of PI, and about 60% are AHU^- (47, 56). Mucosal isolates tend (>60%) to possess the PIB subclass of PI. It is important to note that both PIA- and

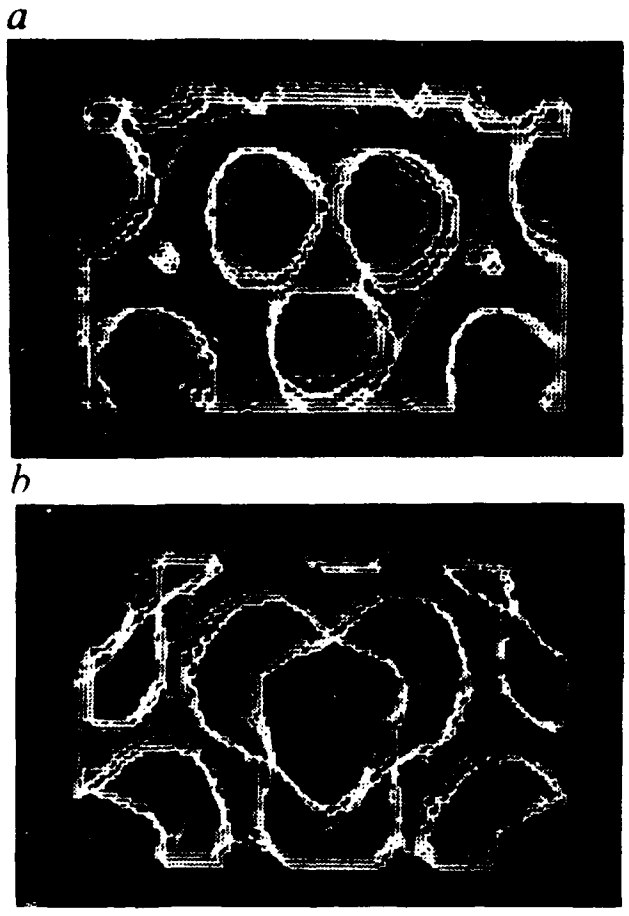

FIG. 3. Three-dimensional reconstruction of the small hexagonal form of a trimeric porin formed by *E. coli* matrix protein (OmpF) in reconstituted phospholipid membranes as viewed externally (a) and internally (b). Conductance measurements demonstrated a series of voltage-dependent openings and closings indicative of a trimeric pore as depicted in panel a. The three pores, which open and close independently, merge to form a single channel on the periplasmic side to the membrane (panel b). Conductance experiments on PI of *N. gonorrhoeae* inserted into lipid membranes demonstrated similar voltage-dependent gating data (51), indicating that PI forms trimeric pores analogous to those formed by the *E. coli* matrix protein as seen here. Reprinted from *Nature* (London) (20) with permission of the publisher.

PIB-bearing strains have been isolated from blood, joints, and mucosal sites (50, 56).

The host immune response to PI depends on previous immune status (28, 66) and site (48, 56) and duration (22, 28, 79) of infection. Several studies have demonstrated that normal human serum contains anti-PI antibody. In one study, normal human serum was shown to specifically inhibit anti-PI monoclonal antibody binding. This inhibiting antibody in normal human serum was opsonic (66). In a pre- and posturethral infection study, immunoblot analysis showed that 12 of 13 men possessed preformed anti-gonococcal antibody, some directed against PI, perhaps being cross-reactive antibody from previous exposure to *N. meningitidis* or other gram-negative organisms (28). Following infection, nine men showed a temporal increase in antibody levels against gonococcal antigens, including PI (28). A study comparing antibodies in serum and vaginal fluid from women with disseminated gonococcal infection, peritoneal inflammatory disease, and uncomplicated mucosal infections indicated that both IgG and IgA in vaginal fluid reacted more strongly with PI of the infecting strain of *N. gonorrhoeae* than did serum antibody (48). Radioimmunoprecipitation studies of serum from sexual partners suffering from localized mucosal infections demonstrated that both men and women produced anti-PI antibody in their sera in response to infection (79).

The role of anti-PI antibody in infection is unknown. However, the possibility that anti-PI antibody is important in limiting infection is supported by the observation that anti-PIII antibody blocked the bactericidal activity of anti-PI antibody in convalescent-phase serum (58), reflecting the close association of PI and PIII in the OM. When anti-PIII antibodies were removed from the serum, bactericidal activity was restored (58), indicating that anti-PI antibody in patient serum can kill *N. gonorrhoeae*.

Many anti-PIA and anti-PIB MAbs are able to activate the classical complement cascade, resulting in cell killing (27, 38, 77). The juxtaposition of the exposed PIA epitopes and other OM components seems to be critical in determining whether a particular MAb will be bactericidal, since MAbs that activate equivalent amounts of complement and that bind equivalently to cells showed marked differences in their ability to kill *N. gonorrhoeae* (38). A similar situation appears to occur with anti-PIB MAbs (77). The difference in killing is related to the manner in which the activated complement is deposited on the bacterial surface (37, 38). Anti-PIA and anti-PIB MAbs have also been shown to be opsonic and are able to inhibit the invasion of *N. gonorrhoeae* into epithelial cells (27).

The ability of anti-PI antibody to kill *N. gonorrhoeae*, combined with the relative abundance of PIs, their universal expression, the apparent structural similarity between PIs within each subclass, their antigenic stability within a strain, and the presence of anti-PI antibody in the mucus and serum, even following uncomplicated mucosal infection, has encouraged the use of PI as a potential vaccine. Purified PIB has been used, unsuccessfully, in vaccine trials (Sexually Transmitted Diseases 1986 National Institute of Allergy and Infectious Diseases Study Group Summary and Recommendations; K. K. Holmes, General Chairman; November 1987) In another study, recipients did show specific responses when injected with a PI preparation (F. Arminjon, M. Cadoz, S. A. Morse, J. P. Rock, and S. K. Sarafian, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, E92, p. 118). Attempts to improve the immunogenicity of PI have been made, especially by using liposomes as delivery vehicles (34). Such studies indicate that both PI-detergent complexes and PI-liposomes induce anti-PI antibodies but that PI-liposomes elicit the larger primary response (34).

The immunodominant portion of the PI molecule appears to be the most exposed region (13, 40, 42, 46, 75). Since this region is also the variable part of the PI molecule, several groups are attempting to identify conserved epitopes which are available to bind anti-PI antibody. One approach is to use MAbs to locate such regions (77). Another approach is to use cloned PI genes to synthesize peptides of PI (13, 14, 23), whereas others are using peptides generated by cleaving purified PIs and recovering surface-exposed fragments for immunogenicity and antigenicity studies (41, 43, 76).

GENETICS

The majority of information about the genetics of PI has come from transformation studies (12). As yet, no mutant

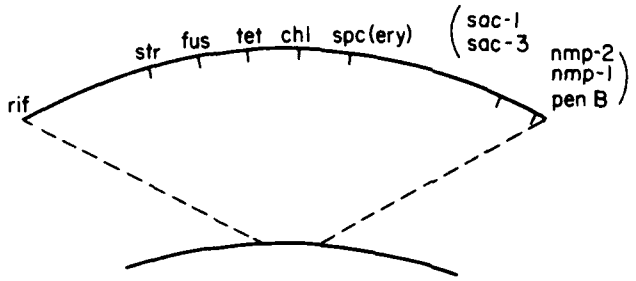

FIG. 4. Linked chromosomal genes for antibiotic resistance (rif, rifampin; str, streptomycin; fus, fusidic acid; tet, tetracycline; chl, chloramphenicol; spc, spectinomycin; ery, erythromycin; penB, nonspecific resistance to penicillin, tetracycline, and chloramphenicol), resistance to serum killing by normal human serum (sac-1 and sac-3), and PI structure (nmp-1 and nmp-2). All loci were mapped by three-factor transformation crosses. Parentheses indicate uncertainties regarding the positions of closely linked markers. Reproduced from *Annual Review of Microbiology* (12) with permission of the publisher.

strains lacking PI have been found, nor have strains been developed which have mutations in a PI gene (13, 14, 23). The availability of PI gene sequences for both PI subclasses (13, 14, 23) may prove invaluable in the generation of PI mutants, providing much-needed information about the structure and function of PI genes and the PI molecule. Transformation, by using both PIA- and PIB-specific MAbs (17), and by using a selectable marker linked to PI genes (13), has already provided several strains of *N. gonorrhoeae* expressing PIA-PIB hybrids, making functional and immunological studies possible.

Transformation studies have identified genetic loci, designated *nmp* (new membrane protein), that affect the apparent molecular mass of PI (10). Introduction of a particular *nmp* results in the expression of a unique PI (e.g., *nmp-1* results in the expression of a PIB-1, *nmp-3* results in a PIB-9, *nmp-4* results in a PIA-1, etc.) (17). The *nmp* loci are very closely linked to loci that influence serum resistance (*sac* [serum antibody complement]) (11) and to the *penB* locus (which is probably identical to the *nsr* locus [nonspecific resistance] described by Bygdeman et al. [7]), which produces low-level, nonspecific resistance to penicillin, chloramphenicol, and tetracycline. The *nmp* region is less closely linked to genes determining resistance to streptomycin (*str*) and spectinomycin (*spc*) and several other antibiotics (Fig. 4) (12). It now appears certain that *nmp* are structural genes for PI (13). Hybridization studies with PIB gene probes suggest that the structural PI gene exists in a single copy in the genome (23). This observation was confirmed by Carbonetti et al. (13), who demonstrated that the PIA and PIB genes are alleles of the same gene.

Transformation usually results in the expression of the donor PI which has the *nmp* locus linked to the *penB* locus (12), although occasionally the recipient PI is expressed (17). This verifies that the *penB* locus can be separated from *nmp*. In a study by Danielsson et al. (17), hybrid PIBs, which acquired unique serovars, were produced in about 6% of the transformants, in which the donor expressed PIB-1 and the recipient expressed a PIB-7, suggesting that recombination had occurred. A similar result was reported for PIA (15), when a transformant generated from a PIA donor and a PIB recipient expressed a PIA that had lost several of the parental PIA epitopes. Moreover, when PIB donor DNA was transformed into a PIA recipient, a single transformant expressed a hybrid PI which had epitopes of both the PIB and PIA molecules (17).

Such observations indicate that new PI molecules, expressing unique surface epitopes, can be generated by genetic exchange between different strains of *N. gonorrhoeae*. The occurrence of PIA-PIB hybrids is extremely rare in nature (46), but the diversity of PI serovars suggests that some form of natural recombination of PI genes does occur. A possible mechanism for in vivo genetic exchange comes from recent observations by Dorward and Judd (18) which demonstrated that naturally elaborated OM blebs of *N. gonorrhoeae* contain DNA. Further, it has been shown that blebs from strains of *N. gonorrhoeae* which possess the gene for penicillinase can transfer penicillin resistance to recipient, penicillin-susceptible strains with great efficiency in the presence of high levels of exogenous deoxyribonuclease (D. W. Dorward, C. F. Garon, and R. C. Judd, submitted for publication). Therefore, in vivo genetic exchange between coinfecting strains of gonococci might occur through bleb-mediated transfer of DNA. PI may form channels between the blebs (which have been shown to possess an abundance of PI molecules) and the recipient cells in a manner analogous to the inverted insertion of PI into eucaryotic cells (3, 49), allowing the DNA to pass from the bleb to the cell.

The close proximity of the *sac-1* and *sac-3* loci to the *nmp* loci may, in an unknown manner, contribute to the relationship of serum resistance and the PIA subclass. The mechanism by which the *sac-1* locus influences serum resistance is unknown, but the *sac-3* locus apparently effects LOS structure (71). Both *sac* loci influence serum resistance independently of the *nmp* loci, since the three loci are separable, and serum-resistant PIB-bearing organisms and serum-sensitive PIA-bearing organisms have been generated by transformation (12, 69). Recent reports of a 29-kDa molecule that correlates with serum resistance and disseminated disease (53) may help elucidate the mechanism(s) of serum resistance and its relationship to PI expression.

The availability of the amino acid sequences for one PIA (14) and two PIBs (13, 23) offers the opportunity to greatly increase our knowledge of the gonococcal PI. Although the PI genes have not been cloned and expressed in a stable cell line (the PIB sequences were elucidated from clones expressing overlapping, truncated PIs, as was the PIA sequence, although an intact PIA was expressed in a short-lived clone), it may now be possible to generate mutations in PI that will greatly improve our understanding of the structure, function, and genetics of PI and hence the role PI in the physiology, pathology, and immunobiology of *N. gonorrhoeae*.

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Molecular Epidemiology of Gonorrhea

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Gonorrhea is a sexually transmitted disease; it is the most frequently reported infectious disease in the United States (12). Humans are the natural host of the etiological agent, *Neisseria gonorrhoeae*, which usually causes uncomplicated infections of mucous membranes of the urethra, cervix, rectum, or pharynx (24). Under certain conditions, the organism may disseminate and cause complicated infections such as pelvic inflammatory disease or disseminated gonococcal infection (DGI) (24). Although the incidence of reported gonococcal infections in the United States has decreased over the past 10 years to approximately 375 cases per 100,000 population, the incidence of infections caused by antimicrobial resistant strains has increased (13).

With the recent development of typing techniques, it has been possible to characterize strains of *N. gonorrhoeae* phenotypically, determine epidemiological correlates of their pathogenicity and antimicrobial susceptibilities, and devise and evaluate strategies to control outbreaks of gonorrhea caused by specific strains. Previously, we have reviewed the applications of phenotypic characterization of *N. gonorrhoeae* strains to studies of reinfection versus treatment failure, coinfection, and forensic investigations; These will not be covered below (34). This review will discuss the methods used to characterize gonococcal isolates and their application to describe strain populations and determine epidemiological correlates of gonococcal infections. The bibliography for this review is extensive but selective and provides key references that will guide the reader to additional readings in this subject.

HISTORICAL PERSPECTIVES ON THE PHENOTYPIC CHARACTERIZATION OF *N. GONORRHOEAE* ISOLATES

Antimicrobial Susceptibility Patterns

Antimicrobial susceptibility patterns (antibiograms) were used to characterize gonococcal isolates before the development of other typing techniques (72). The value of antibiograms alone was limited, however, because they did not permit differentiation between resistant strains that were recently introduced and resistant variants of strains that were already in a community. Nevertheless, in conjunction with other typing methods, antibiograms are essential for the description of the diversity of antimicrobial-resistant strains in communities.

Auxotyping

Auxotyping is the characterization of gonococcal strains according to their nutritional requirements. The method was first developed by Catlin (9). Subsequently, other auxotyping systems have been developed which differ with respect to medium composition and the requirements determined (22, 23, 61). A large number of gonococcal auxotypes have been described (9, 22, 23, 61), and their geographical distribution has been studied extensively (9, 22, 33, 36, 47, 67).

Gonococcal isolates that have no requirements and are designated Zero, prototrophic (Proto), or wild type and those that require proline (Pro) are prevalent worldwide. Arginine-requiring (Arg) isolates are widely distributed geographically but are less numerous; they are rarely isolated in the Far East (31, 34). Isolates with multiple requirements have been isolated. These include, most notably, the arginine-, hypoxanthine-, plus uracil-requiring (AHU) and the proline-, citrulline- (arginine-), plus uracil-requiring (PCU or PAU) isolates.

The AHU isolates, which were isolated infrequently prior to the 1950s (10, 33), were found most frequently in the mid-1970s, when they accounted for as many as 50% of isolates in cities in the United States and Denmark (32, 33, 71). AHU isolates were also frequently found in areas geographically adjacent to the cities in which they were prevalent (36, 40). It has been speculated that the increase in AHU isolates may have been associated with several factors: they may cause asymptomatic infections, resulting in the transmission of infections to many partners; they grow as atypically small colonies; and they may not have been isolated from infected patients on isolation media containing 4 μg of vancomycin per ml (15, 42, 43).

PAU isolates accounted for approximately 40% of isolates in Ontario, Canada, in 1977 to 1978 (23). Similar to the AHU isolates, PAU isolates have been spread to a limited number of geographical areas; they now occur frequently in other cities in Canada, the United States, Europe, and Japan (2, 29, 32, 36, 45). For example, PAU isolates accounted for approximately 5% of all isolates in Seattle and Denver in 1984 (25). Isolates belonging to this auxotype are unusual because they do not possess any plasmids, including the 2.6-megadalton (MDa) cryptic plasmid found in most gonococcal isolates (18).

Strains belonging to certain auxotypes were found to be associated with specific disease syndromes and antimicrobial resistance. AHU isolates were frequently isolated from DGI patients in many cities in the 1970s (19, 31, 43, 63, 65). AHU isolates are serum resistant (59) and highly susceptible to penicillin (31). In the eastern United States, DGI isolates were frequently Proto or Pro (19, 63). However, in contrast to most Proto or Pro strains, DGI isolates belonging to these auxotypes were also highly susceptible to penicillin and were serum resistant (19, 31). These observations resulted in the speculation that non-AHU DGI isolates might be related to the AHU isolates and raised the question of whether another phenotypic typing system, which could be used to characterize large numbers of strains rapidly, might demonstrate a phenotypic relatedness among DGI isolates from different geographical areas. PAU isolates have also been associated with disseminated and asymptomatic gonococcal infections in Winnipeg, Manitoba, Canada. In contrast to the AHU

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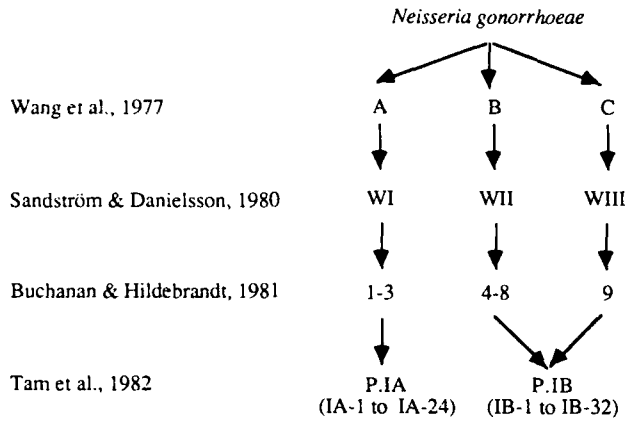

FIG. 1. Historical review of the development of serological classification systems for *N. gonorrhoeae* based on the antigenic specificity of the gonococcal PI molecules.

isolates. PAU isolates are less susceptible to antimicrobial agents (23).

Plasmid Profiles

The plasmids of *N. gonorrhoeae* have been described according to their mobilities in agarose gels. Most gonococcal strains possess a 2.6-MDa cryptic plasmid (41). Five species of β-lactamase plasmids, the 4.4-MDa (Asia), 3.2-MDa (Africa), 2.9-MDa (Rio), 3.05-MDa (Toronto), and 4.0-MDa (Nimes) plasmids, have been identified in penicillinase-producing *N. gonorrhoeae* (PPNG) strains (21, 51, 66, 70). Two conjugative plasmids, a 24.5- and a 25.2-MDa *tetM*-containing plasmid possessed by strains of *N. gonorrhoeae* with high-level resistance to tetracycline (TRNG strains), have also been described (44, 50). Plasmid profiles, in conjunction with auxotyping and more recently with serological classification, have permitted the characterization of isolates from different geographical areas and the documentation of temporal changes in their distribution and prevalence (27, 67).

Serological Classification of Gonococcal Strains

Classification with polyvalent antibodies. Serological typing methods for *N. gonorrhoeae* have been developed and refined during the last decade. Wang et al. (68) developed a microimmunofluorescence test with polyvalent antibodies against formalinized whole gonococcal cells that divided gonococcal strains into three groups, designated A, B, and C (Fig. 1). Subsequently, a coagglutination test permitted Sandström and Danielsson to divide gonococci into three serologically distinct groups, designated WI, WII, and WIII, that corresponded to the Wang serogroups A, B, and C, respectively (56). In 1981, Buchanan and Hildebrandt developed an enzyme-linked immunosorbent assay with partially purified gonococcal protein I (PI) and divided gonococcal strains into nine principal outer membrane protein serotypes (3). Serotypes 1 to 3 corresponded to serogroup WI, and serotypes 4 to 8 and 9 usually corresponded to serogroups WII and WIII, respectively (Fig. 1) (53). Antibodies in both the W serogrouping and POMP serotyping systems reacted with antigens on the gonococcal PI molecule (57). Cross-reactions between WI strains and WII/III-specific serogrouping reagents, and vice versa, were not observed. With

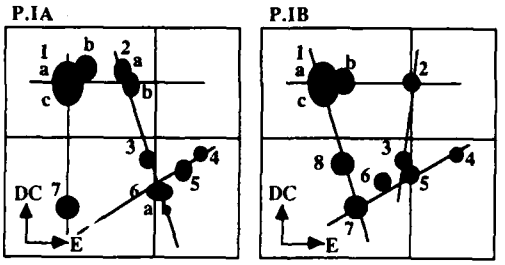

FIG. 2. Graphic representation of patterns of common peptides in strains of *N. gonorrhoeae* that possess either P.IA or P.IB molecules. PI molecules were radiolabeled with ^{125}I and digested with trypsin; the peptides were separated by electrophoresis (E) and chromatography (DC).

two-dimensional peptide mapping of trypic digests of PI molecules, Sandström et al. showed that, with respect to the patterns of common peptides, gonococcal strains produced two types of PI molecules (55). Gonococcal strains belonging to serogroups WII and WIII possessed similar PI molecules, designated P.IB, whereas strains belonging to serogroup WI possessed distinctly different PI molecules, designated P.IA (Fig. 2).

The proportion of isolates belonging to the different W serogroups varied among geographical areas worldwide (7, 16). For example, isolates belonging to serogroup WII were generally isolated significantly more frequently from patients, including homosexual men, than were serogroup WI or WIII isolates (4). Both PPNG and non-PPNG isolates belonging to serogroups WII and III were also generally more resistant to antibiotics than were isolates belonging to serogroup WI (4, 5). Although AHU isolates belonged to serogroup A (WI, serotypes 1 to 3), non-AHU isolates, including those from patients with DGI, also belonged to serogroup WI and could not be distinguished from the AHU isolates serologically (34, 57, 58, 68). The development of monoclonal antibody typing systems permitted differentiation among these isolates.

Serological classification with monoclonal antibodies. Tam et al. (62) developed monoclonal antibodies against gonococcal outer membrane proteins. Antibodies specific for epitopes on P.IA or P.IB molecules were selected by screening against W-serogrouping reference strains in coagglutination tests and confirmed by radioimmune precipitation assays (62).

A standard panel of six P.IA-specific and six P.IB-specific monoclonal antibody reagents was selected (35). Strains characterized serologically by their reaction patterns with these reagents were designated as serovars (35). The nomenclature for this typing system was constructed by using the prefixes IA or IB, indicating which PI molecule was possessed by the strain, followed by a number indicating the pattern of reactions of the strain with the IA- or IB-specific panel of monoclonal antibody reagents. For example, a strain belonging to serovar IB-1 is one that reacts with the P.IB-specific reagents 3C8, 1F5, 2D6, and 2H1 but not with other P.IB-specific reagents. In a study of strains from a worldwide collection, all strains reacted with at least one of these reagents (35). To date, a total of 24 P.IA serovars, designated IA-1 to IA-24, and 32 P.IB serovars, designated IB-1 to IB-32, have been described (Fig. 3). This serological classification system requires the use of the same standard panel of reagents for all studies, a limitation considered vital to permit the global geographical and temporal comparisons of gonococcal strain populations that are essential to moni-

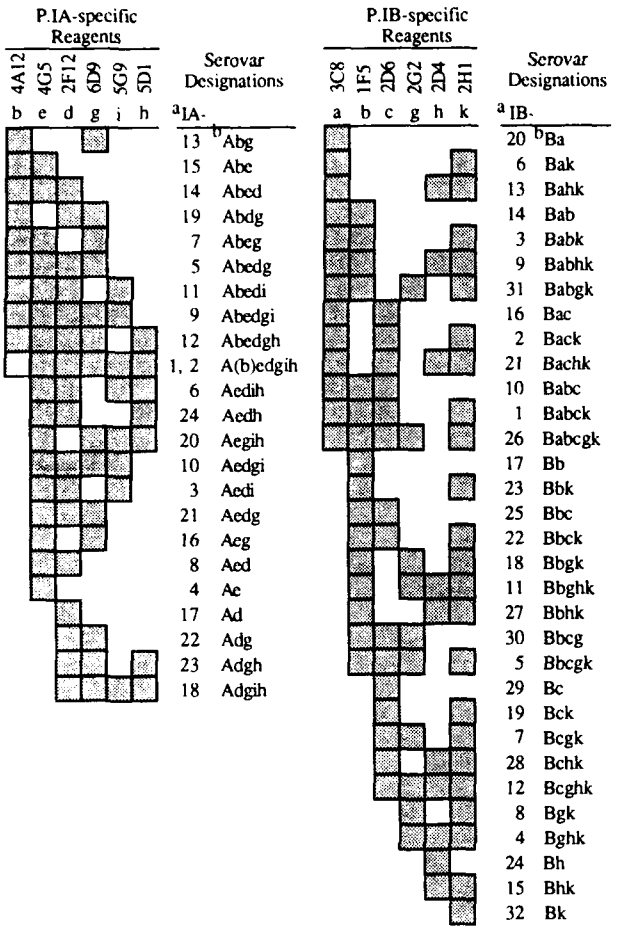

FIG. 3. Reaction patterns and nomenclatures of *N. gonorrhoeae* P.IA and P.IB serovars by using six P.IA-specific or six P.IB-specific monoclonal antibody reagents in a coagglutination test. The shaded squares represent positive reactions with the corresponding reagents. [a] Nomenclature of Knapp et al. (35); [b] nomenclature of Bygdeman et al. (6).

toring the spread of resistant strains. Although the nomenclature for this typing system is inflexible, prevalent serovars can be subtyped with additional reagents, and a specific nomenclature could be devised within the standard nomenclature.

Swedish investigators also devised a serotyping system with the previously developed monoclonal antibodies, which they designated GS antibodies (6, 17). The serovar nomenclature for this typing system uses a combination of upper- and lowercase letters (30) (Fig. 3). The PI expressed by an isolate is designated A or B, and individual monoclonal antibody reagents in the P.IA- and P.IB-specific reagent panels were assigned lowercase letters. Thus, the serovar of a gonococcal strain that reacts with the IA-specific monoclonal antibody reagents e, d, i, and h, is Aedih (30).

Recently, Swedish investigators developed a new set of PI-specific monoclonal antibodies (54) designated Ph antibodies. Antibodies were selected to give patterns of reactions that corresponded to those obtained with the GS antibodies. Similar to the nomenclature for the GS serotyping system, the nomenclature for the Ph serotyping system also used the prefixes A and B; a different set of lowercase letters was used to designate individual reagents (54). These investigators used different panels of reagents to provide greater resolution within a few serovars that were predominant in some populations of patients (6, 8, 17). Unfortunately, the results obtained with different panels of reagents cannot be compared; unless standard panels of antibody reagents are used, it is not possible to make geographical and temporal comparisons of gonococcal strain populations.

Serological classification of gonococcal strains by serotypes. Monoclonal antibody reagents have been used to serotype gonococci; they recognize epitopes of the serotypes 1, 5, 7, 8, and 9 (38). Serotype 9 has been divided into two subtypes, 9a and 9b (38). A particular serotype is referred to by its pattern of reaction with the monoclonal antibody reagents (38). Although the serovar typing systems provide a high degree of resolution, which permits detailed studies of gonococcal strain populations, the serotyping system, by providing less differentiation, may permit a more practical grouping of antigenically related serovars for analyzing antimicrobial resistance patterns that do not require the differentiation provided by the serovar typing systems.

Dual Classification of *N. gonorrhoeae* Strains

A/S classification of *N. gonorrhoeae*. Both auxotyping and serological classification lack the discriminatory power to differentiate among many gonococcal isolates. To overcome the limitations of either of those methods, an auxotype-serovar (A/S) classification system has been proposed (35). This dual classification system, based on two independent phenotypic characteristics that are stable in vitro, provides a greater resolution among gonococcal isolates than does a system based on one phenotypic characteristic. For example, an isolate that requires proline and belongs to serovar IB-4 is assigned to the A/S class Pro/IB-4. The A/S classification of *N. gonorrhoeae* has been used alone (2, 32, 39, 71, 74) or in conjunction with plasmid profiles (37, 71) and antimicrobial susceptibilities to perform detailed analyses of gonococcal strain populations (25).

The A/S classification provides a discriminatory classification system for gonococcal isolates. It must be remembered, however, that this system has several limitations. For example, the proline and arginine requirements of gonococcal isolates may be due to one of several mutations that affect the synthesis of these amino acids (11, 14). Thus, strains belonging to either the Pro or Arg auxotypes may be different; the arginine requirement of isolates has been further defined by the auxotyping methods that test strains for their ability to grow on ornithine or citrulline in the absence of arginine. Similarly, strains belonging to the prevalent serovars such as IB-1 or IB-3 may not be similar, as suggested by the comparison of their antimicrobial susceptibility patterns (34).

Dual serological classification of *N. gonorrhoeae*. A dual serological classification system has recently been proposed (53) in which gonococcal isolates are classified according to their reaction patterns with panels of both GS and Ph antibodies. A strain that reacts with the P.IA-specific GS antibodies e, d, i, and h will react with the Ph antibodies r, s, and t and be assigned to the serovar Aedih/Arst (53).

EPIDEMIOLOGICAL APPLICATIONS OF PHENOTYPIC CLASSIFICATION SYSTEMS FOR *N. GONORRHOEAE*

The classification of *N. gonorrhoeae* isolates by auxotype, serovar, plasmid profiles, and antimicrobial susceptibilities has permitted more detailed analyses of gonococcal strain

populations than was possible by using either auxotype or serovar alone.

Diversity, Regional Differences, and Temporal Changes in Gonococcal Strain Populations

The A/S classification has been used to study the differences among gonococcal strain populations in many geographical areas. A total of 107 different A/S classes were initially identified among more than 1,400 gonococcal strains from North, Central, and South America, as well as from Europe, Australasia, and Africa (35); additional classes have also been recognized by various investigators (25, 32, 39).

The AHU isolates from different geographical areas belonged to the A/S class AHU/IA-1,2 (35); studies have suggested that their distribution has resulted from the spread of a single clone (10). The prevalence of the AHU/IA isolates has decreased since the mid-1970s, and in 1985 they accounted for approximately 15 and 5% of isolates in Seattle and Denver, respectively (25, 32); a similar decrease was observed in Heidelberg, Federal Republic of Germany, between 1981 and 1986 (40).

PAU strains, which previously were geographically limited, have become widespread in the last 10 years (34). Serological classification of PAU strains showed that they belonged to closely related serovars, IB-1, IB-2, IB-10, and IB-16, suggesting that although they may have originated from a single clone, they appear to have undergone some degree of antigenic drift, in contrast to the AHU isolates (2, 32, 71). It should be noted, however, that antigenic drift might also be demonstrable among the AHU strains with the use of additional monoclonal antibodies.

The A/S classification system permitted the differentiation of Proto and Pro isolates into many A/S classes; some of these classes have become widely distributed in different geographical areas, whereas others are limited in that respect (2, 32, 39, 47). For example, strains belonging to the IA serovars IA-5 or IA-9 have been isolated primarily in Africa (34), whereas strains belonging to the IB serovars IB-5 or IB-7 have been isolated in the Far East (34).

Gonococcal Strain Populations in Communities

Gonococcal strain populations in communities may also be composed of many A/S classes; strains belonging to as many as 50 to 60 different A/S classes have been isolated in individual communities (6, 25, 32). Usually, six to eight A/S classes persisted in 30 to 40% of the patients, whereas isolates belonging to most A/S classes were transient, occurring in few patients. Some A/S classes were specifically associated with isolates from heterosexual patients, and others were associated with isolates from homosexual men, suggesting that different strains may be spread within subgroups of the total population at risk for contracting gonorrhea (32). Temporal changes in strain populations in communities have also been demonstrated. Some A/S classes previously associated with heterosexual patients were isolated from homosexual men and vice versa, suggesting a spread between these patient subpopulations as a result of spreading of strains by bisexual men (49). The dynamic nature of gonococcal strain populations appears to be a general phenomenon and is not limited to specific geographical areas (25, 32, 46). The factors that influence the establishment of strains in a community are not clearly understood.

Several readily identifiable selective factors may enhance the ability of strains to persist in a community. These include

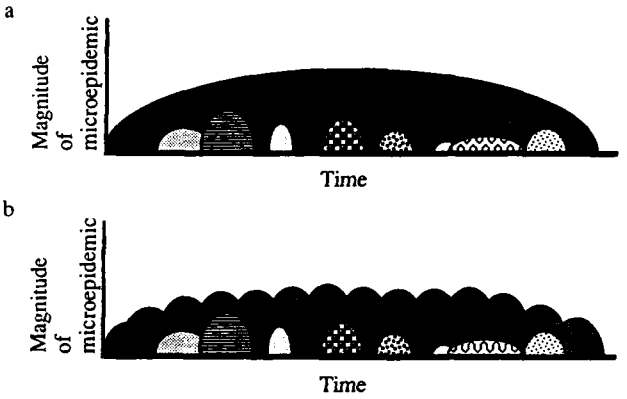

FIG. 4. Graphic representation of *N. gonorrhoeae* strains in a community. (a) The strain population is composed of one persistent strain (large shaded area), extending over the entire period, and a number of transient strains (small shaded areas). (b) It is possible that strains that appear to be persistent actually occur as a series of epidemiologically unrelated outbreaks caused by the same strain.

resistance to antimicrobial agents used therapeutically; hypersusceptibility to vancomycin, resulting in the failure to isolate the organism; and the ability to cause asymptomatic infections, resulting in the failure of a patient to recognize and seek treatment for an infection. Persons with asymptomatic infections may play an important role in spreading infections in a community.

It is equally likely, however, that behavioral factors also play an important role in the introduction and spread of strains in a community, because strains that do not exhibit the selective factors listed above have also become predominant in communities. The frequency at which strains are introduced into communities may affect their prevalence and persistence. Strains may be introduced more frequently from geographically adjacent communities than from distant communities. Consequently, such strains may appear to be persistent in terms of their total numbers and frequency of isolation, whereas they may in fact be persistent as a result of the occurrence of concurrent and sequential microepidemics which are epidemiologically separate events (Fig. 4).

Strains in a community will be most frequently spread by persons with multiple sexual partners, i.e., high-frequency transmitters. These may include not only prostitutes and some homosexual men who have many sexual partners, but also any other person with multiple partners. High-frequency transmitters belonging to the former group may be readily associated with certain geographical locations in communities (such as certain bars) that can be targeted for intensive gonorrhea control programs, whereas persons belonging to the latter group may reside in geographically diverse areas within a community and may not be readily identifiable.

Antimicrobial Resistance in *N. gonorrhoeae*

Antimicrobial resistance in *N. gonorrhoeae* may be chromosomal (20) or plasmid mediated (73). Correlations between antimicrobial resistance and A/S classes have been found. Gonococcal strains that are resistant to penicillin, tetracycline, and the cephalosporins have been isolated (20, 25). A strain belonging to the A/S class Pro/IB-1 and resistant to penicillin, tetracycline, erythromycin, and cefoxitin was isolated during an epidemic of gonorrhea in North Carolina in 1984 (20). Subsequent surveillance studies, how-

ever, showed that strains belonging to several A/S classes exhibited chromosomal resistance to antimicrobial agents (25, 48).

Spectinomycin-resistant *N. gonorrhoeae* strains were first isolated in Korea (1). Since that time they have also been isolated in England and in the United States, where, occasionally, they have also possessed β-lactamase plasmids (26, 74). Most strains isolated in the United States in 1985 to 1986 belonged to the A/S class Pro/IB-1 (74), were chromosomally resistant to several antibiotics, and possessed the 24.5-MDa conjugative plasmid; one isolate belonged to the A/S class Proto/IB-5 and was susceptible to other antibiotics (74).

PPNG strain populations have been analyzed in detail in Miami, Fla., where PPNG infections accounted for approximately 30% of cases of gonorrhea in 1986 (73). The PPNG strain population in Miami has undergone dramatic changes between 1983 and 1986 (75). In 1983, PPNG strains possessing the 3.2-MDa β-lactamase plasmid predominated, and few strains possessed the 24.5-MDa conjugative plasmid. In late 1984, most PPNG strains possessed the 4.4-MDa β-lactamase plasmid. By 1986, most PPNG strains possessed the 3.2-MDa plasmid, and 36% of these also possessed the 24.5-MDa conjugative plasmid. During each period, the PPNG strain population consisted of a few dominant and many transient A/S classes; the predominant A/S classes also differed from one period to the next (75). On the basis of these observations, we speculate that the PPNG strain population in Miami has undergone temporal changes influenced by the introduction and eradication of different PPNG strains rather than by transfer of the plasmids in the community. In view of the prevalence of the 24.5-MDa conjugative plasmid in PPNG strains in 1986, transfer of the β-lactamase and conjugative plasmids to indigenous strains may occur more frequently in the future.

High-level tetracycline resistance conferred on *N. gonorrhoeae* (TRNG) strains is due to the acquisition of a 25.2-MDa *tetM*-conjugative plasmid (44). TRNG isolates have been reported in the United States (37, 44), Canada (60, 64), The Netherlands (52), and England (28, 69) and have been classified by phenotypic typing methods. TRNG isolates belonging to as many as 23 A/S classes have been identified, suggesting that the 25.2-MDa plasmid has been transferred among different gonococcal strains. Similar to other gonococcal strain populations, TRNG strain populations have been characterized by the introduction and eradication of individual TRNG strains, although approximately 50% of isolates initially studied in the United States belonged to the Pro/IB-1 A/S class.

Prospects

We can characterize outbreaks and study epidemiological correlates of gonorrhea by using the phenotypic typing techniques that permit detailed analyses of gonococcal strain populations. Two criticisms of the serotyping system have been voiced: one is that they provide too much discrimination among strains; the second is that more discrimination is required within the major serovars such as IB-1 and IB-3. Future studies will almost certainly include investigations of the relatedness between strains belonging to different serovars and evaluation of additional monoclonal antibodies or other typing techniques to subdivide isolates belonging to the major serovars. Although the current systems have some shortcomings, valuable information concerning the emergence and spread of antimicrobial-resistant strains of *N. gonorrhoeae*, and their association with gonococcal syndromes, can be obtained.

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Animal Models for Pathogenic *Neisseria* Species

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The development and use of various animal models in the early 1970s helped renew interest in the immunobiology of *Neisseria gonorrhoeae* and *N. meningitidis*. This research also provoked controversy concerning which infection model provided the most useful information. Certainly, the human host or isolated organ cultures provided a relevant test system for neisserial infections; however, these models were not accessible to many research groups, and, therefore, alternative models were necessary for preliminary studies. If some discretion is exercised in selecting an appropriate species and in interpreting the results, much useful information can be obtained by studying these sometimes contrived model systems. The question regarding what constitutes a valid model depends largely on the types of experiments being performed. For cell attachment studies, human tissues appear to be essential because of the species-specific nature of this interaction (27). Human organ cultures that may be ideal for studying receptor-mediated attachment may be less suitable for studying the role of humoral factors in pathogenesis because some cell- and complement-mediated systems are not intact. On the other hand, certain animals whose immune systems function in many respects like those of humans can be used to study the complex interaction of the multiple cellular and humoral factors that are involved in the inflammatory response, even though these animals may lack the species-dependent receptors found in tissues of human origin. In this article I will review some of the attributes and deficiencies inherent in the various animal species that have been used as models of infection and point out the aspects of their comparative immunology that are important in interpreting results.

ANIMAL MODELS OF *N. GONORRHOEAE* INFECTION

Some of the earliest published reports of animal infections involving *N. gonorrhoeae* appeared in the late 1930s when Miller described the mouse intraperitoneal and rabbit intraocular models (35, 36). The rabbit model was used briefly for testing the in vivo efficacy of new antimicrobial agents, but the overwhelming success of penicillin in human trials soon thwarted interest in continued animal testing. The next flurry of activity in this area occurred in the early 1970s, when studies of genital infections of chimpanzees (5, 12, 31) and infections of subcutaneous chambers in laboratory animals (1–3, 6, 16) were reported. Results from immunologic studies involving these models heightened interest in the use of animals and in the prospect of developing a successful gonococcal vaccine.

Primate Infection

The chimpanzee (*Pan troglodytes*) is the only animal species other than humans in which localized urethral infections of 3 to 6 weeks in duration have been established (31). Anatomically and physiologically, chimpanzees resemble humans; they have similar ABO blood groups, serum lysozymes, and an immunoglobulin A susceptible to cleavage by gonococcal immunoglobulin A protease (40). Chimpanzees have been infected with laboratory-grown gonococci, and male-to-female transmission has occurred in sexually active cage mates (12). Not unlike the situation in humans, individual variation in susceptibility to gonococcal infection has been observed. Chimpanzees that have urethras colonized by *Proteus* and *Serratia* spp. can show greatly increased resistance to gonococcal infection. In addition, not all human isolates of gonococci are virulent for chimpanzees (4, 30). Nevertheless, chimpanzees have been useful for demonstrating relative, strain-dependent, acquired resistance to *N. gonorrhoeae* following experimental infection or systemic immunization with formaldehyde-fixed cells (4, 5). Interestingly, a greater degree (≥200-fold) of resistance to urethral infection was induced by systemic immunization with formaldehyde-fixed cells than was observed following remission of untreated urethral infection (4, 5, 30). In addition to the enhanced resistance to urethral challenge, immunized male chimpanzees became colonized with relatively fewer gonococci when given an overwhelming challenge dose ($>10^7$ colony forming units), remained infected for a shorter period, and were unable to infect susceptible females (4, 5). Unfortunately, male chimpanzees suitable for gonococcal studies are in limited supply, and their use as a model for human immunodeficiency virus infection will further diminish prospects of their use in other types of research.

Nonprimate Infection

Except for some differences in their comparative immunology, laboratory animal species are readily available for certain types of infection-related experiments. A number of inbred lines of immunologically well-characterized mice are available, and gonococcal infections can be induced in this species by a variety of methods. Mice implanted with subcutaneous chambers are well adapted for graded-dose challenge experiments, which permit the determination of relatively small differences in the virulence of *N. gonorrhoeae* strains or in the acquired resistance of the host. Because some strains of mice are deficient in complement factors necessary for efficient bactericidal activity via the classical pathway, a method has been developed for administering exogenous guinea pig complement at the time of gonococcal challenge. This technique allows some aspects of both complement-dependent and complement-independent resistance to be quantitatively determined (8, 24, 49).

At least two methods for inducing urogenital infections in mice have been reported: inoculation of gonococci into fluid-filled perianal sacs formed by natural or surgically produced strictures of the uterus (11, 51), and transcervical inoculation of gonococci (29). Both methods produce histologic evidence of inflammation, and viable gonococci can be recovered from infected tissues at various days after inoculation. These models offer the advantage that gonococcal infections are induced in a more natural site. However, because murine immunoglobulin A is not susceptible to gonococcal proteases and because the gonococci remain

entrapped in a fluid-filled cavity, these models may not prove suitable for some experiments that examine mucous-membrane attachment, especially when host-specific surface receptors are involved.

Because of their relatively high level of serum complement activity, guinea pigs are often preferred as immunologic models. However, the only reported method for inducing gonococcal infections in this species involves implanted porous chambers (2, 9). The foreign-body effect produced by the subcutaneous chamber appears to create an immunologically privileged site where gonococci can be grown for several weeks before the host immune system terminates the infection. Because small-challenge inocula (10 to 100 colony-forming units) of several gonococcal strains can produce high (>80%) infection rates, guinea pigs are a sensitive model for determining some aspects of acquired resistance induced by systemic immunization (6, 7). By using a graded-dose challenge method, a significant level of strain-related and cross-protective immunity can be quantitatively determined (7, 38, 52). Most strains of gonococci recovered from urogenital infections can infect guinea pigs. Paradoxically, some strains, especially those of the IA-2 serovar often found associated with disseminated gonococcal infections, are not as virulent for guinea pigs (37). This difference may be due to species-related differences in protective host factors such as the availability of metabolic iron, immunoglobulin classes, blocking antibodies, or in S protein, an important inhibitor of terminal complement factors (32, 41).

Rabbits are convenient animals for the production of immune sera, but they are not highly susceptible to subcutaneous neisserial infections unless they are given large doses of dexamethasone, an immunosuppressive drug that inhibits synthesis of interferon by decreasing the level of its messenger ribonucleic acid (23). However, by using a transaortic catheter, one can produce a gonococcal bacteremia in rabbits that results in hepatic lesions similar to those observed in humans (28). Rabbits are generally sensitive to the effects of bacterial endotoxin, and a generalized Shwartzman reaction can be induced by giving two intravenous injections of gonococci 20 to 24 h apart (10). However, attempts to use a rabbit oviduct organ model for studying gonococcal cell attachment have been unsuccessful because only human tissue provides for specific attachment and mucosal damage. Oviducts of bovine and porcine origin were likewise undamaged by gonococci (27).

Laboratory rats are not easily infected with gonococci, probably because of their high level of natural serum bactericidal activity. Rats have been used, however, to demonstrate the arthropathic properties of the gonococcal peptidoglycan that has been implicated in the pathogenesis of disseminated joint disease (20).

Gonococcal infections produced in subcutaneous chambers implanted in hamsters are similar to those described for laboratory mice. Hamsters have a better-developed complement system than do most strains of mice, but their very short tail makes them difficult to handle and increases the risk of the handler's being bitten.

Chicken embryos provide a convenient model for in vivo studies involving some aspects of neisserial pathogenesis (14, 15, 21, 47). However, they lack a complete complement system and are not amenable to active immunization, although the effects of passively transferred antibodies have been tested in this model (19).

ANIMAL MODELS OF MENINGOCOCCAL INFECTION

Various animal models including monkeys, rabbits, guinea pigs, mice, and chicken embryos have been used in the study of different aspects of meningococcal pathogenesis (18, 22, 34, 44). Of these species, the laboratory mouse is probably one of the more versatile animals in terms of methods for inducing infection, because we can select inbred lines with well-characterized immunologic features. The influence of genetic loci on the susceptibility of mice to bacterial invasion is well documented (43). The *Lps* gene locus, which regulates the cellular response to endotoxin, appears to have significant control over the host resistance to meningococcal infection (42, 53). Inbred lines of mice with a defective *Lps* response fail to appropriately activate macrophages during the early stages of infection, resulting in microbial colonization and subsequent hematologic dissemination. However, one must also appreciate that although *Lps*-defective mice can support more rampant bacterial growth, their immunologic and pathologic responses to infection could also be severely compromised.

The mouse intraperitoneal infection model that uses gastric mucin to enhance infection has been used extensively for determining meningococcal strain variation and for elucidating the role of metabolic iron during meningococcal infection (34). Because very little free iron is available to microorganisms growing extracellularly in the human or animal host, many pathogens have developed methods for obtaining essential iron from storage and transport proteins. Both meningococci and gonococci are capable of obtaining iron from transferrin and lactoferrin, whereas most commensal *Neisseria* organisms lack this capacity (33). Holbein found that iron dextran could replace mucin in the mouse intraperitoneal model and suggested that an iron deficiency developed in mice following infection as a result of the failure of transferrin to reload iron (25). Holbein later showed that some virulent strains of meningococci could infect mice independent of exogenous iron, and he suggested that virulence determinants, in addition to iron acquisition, played an important role in pathogenicity (26). Both Miller (34) and Holbein (25) proposed that carrier strains of meningococci may lack the invasiveness of disease strains; therefore, research continued toward developing a relevant infection model that could determine the invasive potential of test organisms.

The mouse intraperitoneal model was shown to have some capability for discriminating between meningococci of high and low virulence. Disease-associated strains generally induced a fatal infection in mice after the injection of relatively few microorganisms (<10 colony-forming units), whereas less virulent or carrier strains had a 50% lethal dose of $>10^4$ colony-forming units (26). Salit (44) described a mouse model in which intranasal challenge was used to test the invasive potential of disease- and carrier-associated strains. Approximately 40% of 2- to 5-day-old mice became bacteremic by 48 to 72 h after intranasal inoculation with virulent meningococci. This model offered an advantage in that meningococcal attachment, mucosal colonization, and invasion of underlying tissue with subsequent hematologic dissemination could be monitored in systematic experiments involving histologic sectioning of the intact mouse.

An infant-rat model for group B meningococci has been developed to evaluate the protective effects of monoclonal antibodies that react with class 1 and class 3 outer membrane proteins (45, 46). Five-day-old Wistar rats were injected intraperitoneally with monoclonal antibodies 1 or 20 h before

the intraperitoneal injection of 10^5 colony-forming units of a group B meningococcal strain. These monoclonal antibodies gave significant protection against the subsequent bacteremia, meningitis, and death that occurred in the control animals. Peppler and Frasch previously showed that guinea pigs could be protected against group B meningococci by immunization with a serotype 2 vaccine (39). These models are convenient tools for evaluating the systemic effects of various immunologic interventions. Additional factors that are important in the human disease, however, such as the effects of immunologic maturity of the host, the role of mucosal attachment, and tissue invasion, must also be considered if an appropriate model is to be developed.

ORGAN CULTURE MODELS

The human fallopian tube organ culture has provided researchers with an excellent system in which to study the gonococcus-host cell attachment and the localized spread of microorganisms. In this model, the initial attachment of gonococci to nonciliated host cells appeared to be mediated by the interaction of gonococcal pili with microvilli of the mucosal cells (27, 48). The release of cytotoxins, including lipopolysaccharide and monomeric peptidoglycan, resulted in injury to the host cells as evidenced by the loss of ciliary activity and eventual sloughing of the cells. Important questions concerning tissue penetration by gonococci and the effects of immunoglobulin A protease have been addressed by using organ culture (17). Endocytosis and phagocytosis of gonococci by host cells appear to play major roles in the translocation of gonococci to the subepithelial space in organs where the bacteria can multiply.

Another organ culture, containing human nasopharyngeal tissue, has been used to evaluate the interaction of *N. meningitidis* with mucous membranes. Meningococci selectively attached to the microvilli of nonciliated epithelial cells and were internalized by endocytosis. Although meningococci failed to attach to ciliated cells, ciliary function and cell viability were damaged by lipopolysaccharide released by the microorganisms (48).

CONCLUSIONS

Although many successful experiments have been reported, the use of various animal models to test the virulence and immunogenicity of *Neisseria* spp. remains controversial. Animal trials with chimpanzees, guinea pigs, and mice have shown that significant immunity, either relative or absolute, can be induced by systemic immunization with formaldehyde-fixed cells or with protein outer membrane complex. No increased resistance was obtained, however, by administering preparations of highly purified gonococcal pili (13, 50). The relative degree of cross-protection afforded by immunization with different neisserial strains has also been shown in these models. Surprisingly, results of gonococcal immunization trials in these species appear to be consistent, even though the routes of infection, intraurethral for chimpanzees and subcutaneous in mice and guinea pigs, are very different. The ability to test various immunogens in animal models has allowed researchers to establish that systemic immunization can affect strain-related resistance to *Neisseria* spp. and should continue to provide an alternative method for preliminary testing of potential vaccines.

Many unanswered questions exist concerning the attachment and in vivo growth of pathogenic *Neisseria* spp. The role of pili is not completely understood. The nature of the receptors that mediate specific attachment of cells and lipopolysaccharides also awaits further definition. The species-specific binding of *N. gonorrhoeae* and *N. meningitidis* with host proteins, including transferrin, lactoferrin, and S protein, is being studied in an attempt to isolate cell components that could have vaccine potential. The preliminary testing of these candidate vaccines will probably involve animal studies. Therefore, it is essential that investigators select the most appropriate model, based on current information concerning the pathogenic mechanism of infection and the type of vaccine intervention being tested.

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Protein III: Structure, Function, and Genetics

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Protein III (PIII) was originally described by McDade and Johnston (14). In their studies of the gonococcal outer membrane, they observed that all gonococcal strains contained a similar protein whose apparent molecular weight in sodium dodecyl sulfate-polyacrylamide gel electrophoresis increased upon treatment with a reducing agent. Furthermore, they and others (14, 17) provided evidence, using bifunctional cross-linking reagents, that PIII was closely associated with the protein I (PI) porin within the gonococcal outer membrane. It was further shown that PIII was structurally and immunologically conserved among several strains of gonococci (11, 12). In studies in which several proteolytic enzymes were used to investigate the surface orientation of gonococcal outer membrane proteins, PIII was found to be resistant to protease treatment (2). However, Shafer and Morse, using lysosomal cathepsin G, showed that PIII in gonococcal outer membranes could be cleaved by this enzyme (20). In addition, the rate at which PIII was degraded by cathepsin G could be correlated to the lipooligosaccharide expressed by a particular strain of gonococcus; i.e., the PIII associated with low-molecular-weight lipooligosaccharide was cleaved more rapidly than was the PIII associated with a higher-molecular-weight lipooligosaccharide. Surface-labeling experiments and the ability of PIII to react in vivo with a monoclonal antibody also indicated that PIII had surface-exposed domains (22). Our interest was piqued by several unanswered questions. We wanted to know (i) how this highly conserved, surface-exposed protein could exist in an organism that expended a great deal of its genome and energy in antigenic variation; (ii) how, if at all, PIII affected the functions of PI; and (iii) how PIII could be eliminated as a contaminant in preparations of PI.

PURIFICATION

To study PIII more directly, we developed a method for purifying it (13) and began to examine its biochemical and immunochemical characteristics. Early on, we observed that to purify PI, it was essential to separate PI from PIII in the initial extraction step. Otherwise, PIII remained tightly associated with PI throughout the subsequent steps of purification. We found that if the pH of the extraction buffer was altered, different proteins would be solubilized. If the extraction was performed at pH $\leq$ 4, PI and PII were quantitatively released, but PIII remained with the cell debris (1). PIII was solubilized in rather pure form by reextraction of the cell debris with a pH 10.5 buffer. It was completely released with minimal PI contamination. The PIII was then purified by cation-exchange chromatography on CM-Sepharose, followed by gel filtration on Sephacryl-200 (13). The resulting product retained all the biochemical and immunochemical characteristics of the native PIII, except that, unlike the native molecule, purified PIII was highly susceptible to proteases. The amino acid composition and amino-terminal sequences of PIII isolated from several strains of *Neisseria gonorrhoeae* were determined and found to be identical. Furthermore, these data suggested that PIII was distinct from gonococcal PI or PII. Polyclonal rabbit antiserum was raised to the purified PIII and used in whole-cell enzyme-linked immunosorbent assay (ELISA) inhibition studies. These data confirmed the results of others (12, 22), which suggested that PIII was surface exposed and indicated that approximately 70% of the antibodies to the purified molecule were adsorbed by intact gonococci. It was also observed that the class 4 protein of *N. meningitidis* was recognized by these antisera.

CLONING OF THE STRUCTURAL GENE

The structural gene of PIII has been cloned in the expression vector λ gt11 (7). Unlike clones of gonococcal PI, the cloned PIII gene produced a full-sized, immunologically reactive product in *Escherichia coli* following isopropyl-β-D-thiogalactopyranoside (IPTG) induction as shown by Western immunoblot analysis. The cloned protein displayed the typical increase in apparent molecular weight in sodium dodecyl sulfate-polyacrylamide gel electrophoresis upon reduction. The PIII gene has been sequenced by the chain termination method and found to contain an open reading frame of 236 amino acids (8). This consisted of a typical 22-amino-acid signal peptide sequence followed by the known N-terminal sequence. However, the calculated molecular weights for the pro-protein and the mature protein were 25,544 and was 23,298, respectively. This was almost 8,000 less than expected from its migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. There were several lines of evidence indicating that the PIII sequence terminated at nucleotide 813 (rather than at about nucleotide 1000). First, a subclone in plasmid pUC9 extending from nucleotides 1 to 897 produced a complete PIII product. Second, a typical transcription terminator was found starting at nucleotide 909. Third, the predicted amino acid composition of the mature PIII corresponded closely to that determined by analysis. Several different methods have been used in an attempt to determine the carboxy-terminal residue of gonococcus-expressed PIII by using carboxypeptidase Y digestion (E. J. Lytton and M. S. Blake, unpublished data). No free amino acids were released, suggesting that the carboxy terminus was blocked or unavailable for cleavage.

Recently, Klugman (personal communication) has cloned and sequenced the gene of the *N. meningitidis* class 4 protein by using similar cloning and sequencing strategies that were used with PIII. His results showed that the sequences of the two proteins (PIII and class 4) were almost identical, the most pronounced differences being within the proline-rich area.

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proOmpA  MKKTAIAIAVALAGFATVAQAAPKDNTWYTGAKLGWSQYH
                10        20        30        40
proOmpA  DTGFINNNGPTHENQLGAGAFGGYQVNPYVGFEMGYDWLG
                50        60        70        80
proOmpA  RMPYKGSVENGAYKAQGVQLTAKLGYPITDDLDIYTRLGG
                90       100       110       120
                     10        20        30
proPIII       MTKQLKLSALFVALLASGTAVAGEASVQGYTVSGQS

proOmpA  MVWRADTKSNVYGKNHDTGVSPVFAGGVEYAITPEIATRL
               130       140       150       160
           40        50        60        70
proPIII  NEIVRNNYGECWKNAYFDKASQGRVECGDAVAVPEPEPAP
                                                :..:.
proOmpA  EYQWTNNIGDAHTIGTRPDNGMLSLGVSYRFGQGEAAPVV
               170       180       190       200
           80        90       100       110
proPIII  VAVVEQAPQYVDETISLSAKTLFGFDKDSLRAEAQDNLKV
         ... ..:::.  .. ..:.....:::.:.:...:.:.:.:.
proOmpA  APAPAPAPEVQTKHFTLKSDVLFNFNKATLKPEGQAALDQ
               210       220       230       240
          120       130       140       150
proPIII  LAQRLSR--TNVQSVRVEGHTDFMGSEKYNQALSERRAYV
         :  .:::.  ..  :: : :.:: .:::. :::.:::::::
proOmpA  LYSQLSNLDPKDGSVVVLGYTDRIGSDAYNQGLSERRAQS
               250       260       270       280
             160       170       180       190
proPIII  VANNLVSNGVPASRISAVGLGESQAQMTQVCQAEVAKLGA
         :.. :.:.:.::..::: :.::::..  ...:
proOmpA  VVDYLISKGIPADKISARGMGESNPVTGNTC---------
               290       300       310
             200       210       220       230
proPIII  KASKAKKREALIACIEPDRRVDVKIRSIVTRQVVPARNHH
           ...:.:.:::.:..:::::::.....:
proOmpA  --DNVKQRAALIDCLAPDRRVEIEVKGIKDVVTQPQA
               320       330       340

proPIII  QH
```

FIG. 1. Comparison of the amino acid sequences of *N. gonorrhoeae* PIII (8) and *E. coli* OmpA (4). Homology between the two proteins is indicated by a colon, and conservative substitutions are indicated by a period. Furthermore, each of these two proteins has been compared with the sequence of *P. aeruginosa* protein F (6), and where their respective sequences compare significantly, the sequence is underlined.

PARTIAL HOMOLOGY WITH PROTEINS FROM OTHER GRAM-NEGATIVE ORGANISMS

The translated sequence of PIII was compared with those of other known protein sequences and found to have homology with enterobacterial OmpA proteins (3). PIII also has significant sequence homology with the protein F gene of *Pseudomonas aeruginosa* (6). (Fig. 1). The similarity between OmpA and PIII begins in a proline-rich region, where OmpA has five proline residues interspersed with valines or alanines. The same feature is seen in PIII, except that in addition to the six valines or alanines, the area also contains three glutamates and one glutamine. Thereafter, the homology is very striking, with the exception of the terminal 14 amino acids in PIII. The distance between the homologous cysteine residues is 11 amino acids longer in PIII. In the nonhomologous region of PIII, just to the amino-terminal end of the proline-rich region, there are two cysteine residues at positions 47 and 63. This disulfide loop is not seen in any of the other known OmpA proteins. However, in the *P. aeruginosa* protein F sequence, there is an area containing four cysteines just following the proline-rich region (6). We were interested in how these cysteines related to each other, and we took advantage of two methionyl residues at positions 139 and 182 to determine whether the two cysteine residues at positions 186 and 209 were disulfide bonded to those at positions 47 and 63. Under nonreducing conditions the molecule was chemically cleaved by cyanogen bromide at the methione residues (9). These fragments were then analyzed by gel electrophoresis, by the methods of Swank and Munkres (21). When cyanogen bromide-generated PIII fragments in a nonreduced form were compared with those which had been reduced prior to electrophoresis, no difference was seen and the number of fragments observed was the same. This suggested that the cysteine residues at positions 47 and 63 formed one 15-amino-acid disulfide loop, while the cysteine residues at positions 186 and 209 formed another. Likewise, we have compared the cyanogen bromide-generated fragments from the purified cloned product with those generated from the purified gonococcal product. All the peptides had similar migrations, with the exception of one in each case. The peptide from the gonococcal PIII was isolated by using reverse-phase high-pressure liquid chromatography on a diphenyl column and subjected to amino acid sequencing. This revealed the sequence beginning at residue 183; it also showed that this peptide contained the carboxyl terminus. The observation that the peptide fragment from the gonococcal PIII migrated with an apparent molecular weight that was approximately 2,000 higher than that of the fragment from the cloned product, as well as our inability to obtain a free residue upon carboxypeptidase Y digestion, suggests that an additional substance may be covalently bound to the carboxyl terminus. Such a terminal addition does not seem to be the case with the *E. coli* OmpA protein. However, the carboxyl terminus of PIII diverges at position 224 from the sequence of the *E. coli* OmpA.

PIII STRUCTURE

Morona et al. (15, 16) studied the surface-exposed regions of the OmpA protein of *E. coli*. Utilizing several bacteriophages which use OmpA as a receptor (5, 23), they obtained a large number of phage-resistant OmpA mutants. From the changes in the sequence of the OmpA gene in these mutants (15, 16) and by comparison of enzymatic cleavage of OmpA between intact organisms and cell envelopes (4), a model of the OmpA protein within the membrane has been proposed (15). Changes in the OmpA sequence in the phage-resistant mutants were clustered in four major areas, which could be envisioned as four surface-exposed loops. A large periplasmic portion of the protein that begins at about residue 198 of OmpA is predicted from the proteolytic resistance of OmpA, except when studied in isolated envelopes. In this case, proteolytic cleavage occurs at residue 198 of OmpA. Thus, the homology of the PIII sequence with that of OmpA begins where it is postulated that the OmpA molecule is within the periplasmic space. If the analogy and the model of the two proteins is in fact the case, only residues 23 to 70 would be exposed on the surface of the gonococcus, while the rest of the PIII molecule would be periplasmic. This might explain the resistance of PIII to proteolysis in intact organisms and the drastic change in this resistance during the purification process. If, however, PIII was located in the membrane such that the homologous portion extended externally, antibodies reactive to PIII might occur through exposure to *E. coli* rather than to *N. gonorrhoeae* or *N. meningitidis*. Recently, we used a whole-cell ELISA inhibition study to determine which of these two models applies to PIII. The results of such an experiment are shown in Fig. 2. These data would suggest that at least 40% of the PIII-OmpA cross-reactive antibodies were removed by incubation with intact gonococci. Other data substantiated the hypothesis that portions of the OmpA-like region on PIII were exposed on the surfaces of gonococci (18). Using the primary structure of PIII, we are proceeding to map the surface-exposed portions by immunological and biochemical techniques.

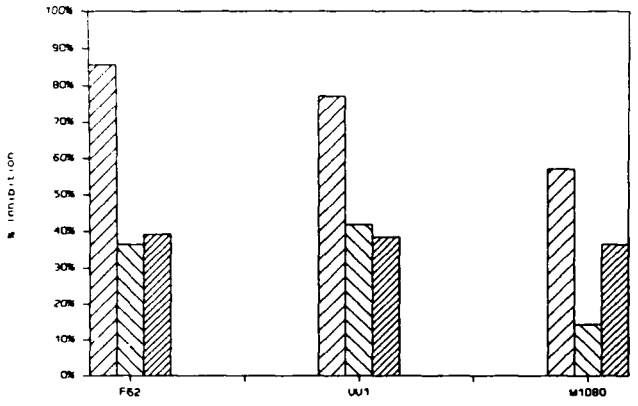

FIG. 2. Whole-cell inhibition ELISA of the reactivity of two hyperimmune PIII antisera (and) and one OmpA antiserum ( ) with purified OmpA. The basis of this ELISA has been described previously (13). Microdilution plates were coated with purified OmpA. Each of the hyperimmune rabbit antisera was then diluted with phosphate-buffered saline to obtain a titer for each serum sample which would give a reading of 1.0 at 405 nm in the ELISA. Bacteria from two different gonococcal strains, F62 and UU1, and one meningococcal strain, M1080, were suspended and diluted in phosphate-buffered saline until the optical density at 500 nm reached 0.6. This bacterial suspension of each strain was dispensed into 0.1-ml aliquots and centrifuged, and the supernatant was removed. The bacteria were suspended in 0.1 ml of the above-mentioned dilution of each serum and incubated for 2 h at 4°C. The bacteria and adsorbed antibodies were then removed by centrifugation. The supernatant containing the antibodies which did not adhere to the bacteria was then applied to microdilution plates which had been precoated with purified OmpA. The rabbit antibodies were then detected by ELISA with a goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. The percent inhibition was calculated as follows: percent inhibition = [1 − (ELISA value of sera after whole-cell absorption)/(ELISA value of sera not absorbed)] × 100.

PIII FUNCTION

The function of PIII either in pathogenesis or in the physiology of the organism is unknown. There is also no information about the effect of PIII antibodies on the infection at either a mucosal or a systemic level. However, Rice and co-workers (10, 19) showed in very carefully controlled in vitro studies that human complement-fixing immunoglobulin G (IgG) antibodies to PIII interfered with the bactericidal activity of immunoglobulin G antibodies directed to other surface antigens such as lipooligosaccharide. These aspects are described more fully elsewhere in this issue (18). These observations, i.e., that antibodies to PIII block bactericidal antibodies to other gonococcal surface structures, might explain why PIII is so highly conserved, but they raise numerous other questions. Among these are how these naturally occurring blocking antibodies arise. For example, are they elicited by OmpA-bearing *E. coli*, by other neisserial infections, or possibly by both? What are the epitopes recognized by the blocking immunoglobulin G antibodies? How do these antibodies contribute to the pathology of gonorrhea?

MUTANTS LACKING PIII EXPRESSION

Recently, a mutant lacking the PIII protein in the outer membrane was obtained (Wetzler, Gotschlich, and Blake, unpublished data). With these mutants and others presently being constructed within our laboratory, we hope to more fully determine (i) the function of PIII; (ii) epitopes of PIII which are surface exposed; and (iii) the way in which PIII and antibodies to PIII affect the pathogenesis of gonococcal infections. Furthermore, with gonococcal mutants lacking in expression of PIII, we can now purify and more fully understand PI without potential PIII contamination.

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Unified Nomenclature for Pathogenic *Neisseria* Species

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The pathogenic members of the genus *Neisseria* include *Neisseria gonorrhoeae* and *N. meningitidis*. Both organisms are obligate pathogens of humans; both colonize nonenteric mucosal surfaces; and both cause communicable infections that can appear as either overt, silent, or chronic infections. On the crude level of deoxyribonucleic acid (DNA) hybridization, these gram-negative diplococci share considerable DNA homology. A more detailed pattern of interspecies relatedness emerges when one examines the various protein and nonprotein constituents of these bacteria. Recently, a number of genes have been cloned and sequenced, and the relatedness of these species has been confirmed on the basis of DNA and predicted protein sequences.

UNIFIED NOMENCLATURE

At the Sixth International Conference on Pathogenic Neisseriae (October 1988), a nomenclature workshop was held. The purpose of the workshop was to revitalize a previously established process whereby neisseriologists might consider genetic and gene product nomenclature and reach a consensus (2). The nomenclature committee opted to follow the course that was taken by the *Escherichia coli* K-12 geneticists a number of years ago. Recommendations were made for genetic nomenclature in line with the rules of Demerec et al. (1); it is assumed that gene product designations will evolve to be the same as the gene designations.

TABLE 1. Old and new symbols for genes and gene products of *N. gonorrhoeae* and *N. meningitidis*[a]

Old symbol				New symbol (both species)		Description
N. gonorrhoeae		*N. meningitidis*		Gene product	Gene	
Gene product	Gene	Gene product	Gene			
Protein I (PI)	*nmp*	Class 1		Por, PorA[b]	*por*, *porA*	Major outer membrane protein; porin
		Class 2 and 3		PorB[c]	*porB*	
Protein III (PIII)		Class 4		Rmp	*rmp*	Reduction-modifiable protein
Protein II (PII)	*opa*	Class 5		Opa_{strain}	opa_{strain}, e.g., $opaI_{F62}$	Opacity-related proteins, also heat-modifiable proteins
Pilin	*pilE1*, *pilE2*	Class I pilin Class II pilin		Pilin	*pilE1*, *pilE2*	*pilE*—expressed locus for pilin structural gene
	pilS				*pilS*	*pilS*—silent (nonexpressed) locus for pilin structural gene
Anaerobically induced proteins				Ani	*ani*	Anaerobically induced proteins
Oxygen-induced proteins				Oxi	*oxi*	Oxygen-induced proteins
H.8 antigen (H8)		+[d]		Lip	*lip*	Lipoprotein
Azurin		+		Laz	*laz*	Lipid-associated azurin
Iron-binding protein		+		Fbp	*fbp*	37-kDa iron-binding protein
Iron-regulated protein		+		Frp	*frp*	Iron-regulated protein
Heat shock protein		+		Hsp	*hsp*	Heat (or stress)-induced proteins
Lipopolysaccharide/ lipooligosaccharide		+		LPS	*lps*	Lipopolysaccharide (macromolecular complex of lipid A and covalently bound sugars)
Iron uptake deficient		+		Fud	*fud*	Iron uptake deficient
–	–	Meningococcal capsule		Cps	*cps*	Meningococcal capsule biosynthesis genes
Transferrin receptor	–			Trd	*trd*	Transferrin receptor deficient

[a] Participants in the Nomenclature Workshop, Sixth International Conference on Pathogenic Neisseriae were as follows: Michael A. Apicella, Department of Medicine and Microbiology, State University of New York, Buffalo, NY 14215; Janne G. Cannon, Department of Microbiology and Immunology, University of North Carolina School of Medicine Chapel Hill, NC 27599; Virginia Clark, Department of Microbiology and Immunology, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14624; Carl Frasch, Center for Drugs and Biologics, Bethesda, MD 20892; Emil C. Gotschlich, Department of Microbiology, Rockefeller University, New York, NY 10021; John Heckels, Department of Microbiology, Southampton University Medical School, Southampton SO9 4XY, Hampshire, England; Penny J. Hitchcock, Department of Microbiology and Immunology, University of Tennessee, Memphis, TN 38163; Harry Jennings, Division of Biological Sciences, National Research Council, Ottawa, Canada K1A OR6, Zell McGee, Department of Medicine, University of Utah School of Medicine, Salt Lake City, UT 84132; Stephen A. Morse, Sexually Transmitted Diseases Laboratory Program Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA 30333; Jan Poolman, Laboratory of Medical Microbiology, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands; P. Fred Sparling, Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC 27599; John Swanson, Laboratory of Microbial Structure and Function, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, MT 59840; and Wendell Zollinger, Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, DC 20307.

[b] *N. gonorrhoeae*, Por and *por*; *N. meningitidis*, PorA and *porA*.

[c] *N. meningitidis*, PorB and *porB*.

[d] An analogous product has been identified in *N. meningitidis*.

differing from the gene by the use of a capital letter (OmpA, gene product) compared with lower-case italics for the gene symbol (*ompA*, gene).

Table 1 includes the old and new symbols for the genes of *N. gonorrhoeae* and *N. meningiditis*. Most of these symbols are self-explanatory, with four exceptions. First, the major outer membrane proteins (porins) have been designated Por. In gonococci, the two types of protein I (PIA and PIB) appear to be gene products of two alleles of the gene *por*; in meningococci, two genes are expressed simultaneously. One gene product is a class 1 protein, now PorA, and the other is either class 2 or class 3, now PorB. Second, the opacity-related proteins, Opa, will require a strain designation to clarify a gene designation. For instance, *opa1* will occur in strains JS3, MS11, and FAM1090; each gene is distinct and will be identified as $opa1_{JS3}$, $opa1_{MS11}$, $opa1_{FAM1090}$, etc. Third, the genetics of the meningococcal class I and class II pilin proteins are undefined, and the current designations of *pilE* and *pilS* may be inadequate in meningococci. Finally, the designation of LPS instead of LOS reflects the consensus reached by the participants in the workshop. It was agreed that the genetic symbol *lps* would be used and that an author's preference for LPS or LOS as a gene product designation would be accepted.

The nomenclature described will be incorporated into the genetic linkage map of *N. gonorrhoeae* and into a detailed review of the genetic loci and linkage associations of *N. gonorrhoeae* and *N. meningitidis* (3). Furthermore, it is the hope of this committee to establish a policy of review at each Pathogenic Neisseriae Conference to maintain a unified nomenclature that complies with the guidelines of Demerec et al. (1).

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Structure and Function of Pili of Pathogenic *Neisseria* Species

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PILI AND THEIR ASSOCIATION WITH VIRULENCE

Pili are hairlike filamentous appendages which extend several micrometers from the bacterial surface and have long had an important role in the pathogenesis of gonococcal infections. Pioneering studies by Kellogg et al. demonstrated that a loss of virulence was observed when gonococci were subjected to repeated laboratory subculture and that this change was associated with a change in colony morphology of the bacteria when grown on solid media (21). Four characteristically different colonial forms, designated types T1, T2, T3, and T4, could be observed; primary isolates produced predominantly small, domed, highlighted colonies (T1 and T2), whereas the laboratory subculture resulted in an increasing proportion of large, flat colonial forms (T3 and T4). Each type could, however, be stably maintained by careful colony selection during subculture, and T1-T2 colonial forms retained their virulence for human volunteers (20). Subsequent electron-microscopic studies revealed that the T1-T2 colonial forms produced pili, whereas the T3-T4 colonial forms did not (54). This association between piliation and virulence prompted a considerable body of work on the structure, function, immunochemistry, and genetics of gonococcal pili and, subsequently, related studies of meningococci.

PILUS STRUCTURE

Subunit Structure

Pili can be obtained from gonococci by utilizing their ability to form crystalline aggregates under appropriate ionic conditions. Repeated cycles of disaggregation followed by crystallization can produce pilus preparations of high purity (2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of such preparations from both gonococci and meningococci revealed the presence of a predominant polypeptide, pilin, with a molecular weight which varied between strains in the range 17,000 to 22,000 (2, 5, 38). This led to the concept that pili were entirely composed of a repeating array of identical pilin subunits. Improved resolution on sodium dodecyl sulfate-polyacrylamide gel electrophoresis has revealed that pilus preparations may contain two closely moving bands representing heterogeneity of the pilin protein (34; J. E. Heckels and P. R. Lambden, unpublished observations). Even greater heterogeneity can be observed when pilin preparations are subjected to isoelectric focusing (22). It is unclear whether such heterogeneity is due to minor modifications such as deamination of glutamine and asparagine residues or truly represents the presence of distinct pilin molecules. Recently, it has also been suggested that pilus preparations may contain minor amounts of accessory proteins which could play an important functional role (31) similar to the tip-located pilus proteins of uropathogenic *Escherichia coli* (26).

The primary sequence of pilin molecules from gonococcal strains R10 and MS11 were first determined by Schoolnik et al. (42), who found by using protein sequencing that pilin molecules contain approximately 160 amino acid residues with methionine at positions 7 and 92 and a single disulfide loop occurring between residues 140 and 151. Cleavage at the methionine residues produced three peptides, CNBr-1 (comprising the first 7 residues from the N terminus), CNBr-2 (residues 8 to 92), and CNBr-3 (residues 93 to 159). By comparing the sequences of the fragments from each strain, the authors suggested that the CNBr-2 region was conserved between strains, whereas the carboxy-terminal CNBr-3 showed significant differences, which were responsible for antigenic differences between strains (39). This model has subsequently been refined by sequence analysis of cloned pilin genes from both inter- and intrastrain pilus variants (see below).

Meningococcal Pilins

Pili isolated from meningococci by the disaggregation methods outlined above have been found to have a similar morphology and composition to those of gonococcal pili with subunit M_r in the range 17,000 to 21,000 (33, 49). Amino acid sequencing revealed significant N-terminal homology with gonococcal pili through to residue 50 (17, 33), and antibodies raised against the CNBr-2 peptide of gonococci cross-react with the meningococcal pili (49), suggesting considerable similarities between the two species.

In a subsequent study, two monoclonal antibodies (SM1 and SM2) which recognize conserved epitopes in all gonococcal pili so far examined (69) were used to detect piliation in meningococcal isolates. A large proportion failed to react with both antibodies but were nevertheless shown by electron microscopy to be piliated (9). These strains also failed to react on Western immunoblots with polyclonal antisera raised against gonococcal pili, but did react with the same sera on immune precipitation under nondenaturing conditions, to reveal pilins with M_r in the range 13,000 to 16,000. Recent studies with synthetic peptides have located the epitopes recognized by antibodies SM1 and SM2 to separate epitopes located in CNBr-2 and CNBr-3, respectively. Thus, meningococci express one of two quite distinct classes of pili: class 1 pili closely resemble gonococcal pili, whereas class 2 pili are composed of smaller pilins which lack epitopes in both the CNBr-2 and CNBr-3 regions but do contain conformational determinants which are shared with class 1 and gonococcal pili. The genes encoding expression of the class 1 pili have been cloned and sequenced, confirming the similarity with gonococcal pili (35a), but the detailed structure of the class 2 pili is not yet known. Clearly, the distinct structural differences between the two types of meningococcal pili may have important implications for their role in pathogenesis.

Quaternary Structure

The way in which pilin monomers fold and assemble to form the quaternary structure of the actual pilus is not yet

known. One model based on a combination of methods of secondary structure prediction and protein homology has suggested that pilin contains four antiparallel α-helices similar to tobacco mosaic virus coat protein (7). Preliminary X-ray-crystallographic analysis of pilus crystals appears to confirm the model and should ultimately provide information on how the pilin subunits assemble into the mature pilus structure (34). Such information should provide valuable information in identifying those functionally important domains of the pilus which interact with the host.

ROLE OF PILI IN PATHOGENESIS

Adhesion to Mucosal Surfaces

The ability of gonococci to attach to the mucosal surfaces of the genital tract and to multiply there despite the flow of mucus and other body fluids is the essential first stage in the pathogenesis of gonorrhea. Subsequent events include uptake by nonciliated columnar epithelial cells and intracellular multiplication, followed by invasion of subepithelial connective tissues (74). Following the observation that virulent colonial forms of gonococci differed from avirulent forms in their possession of pili, many studies have demonstrated that pili facilitate adhesion to a wide range of different cell types. Thus, Pil$^+$ gonococci show an advantage over Pil$^-$ variants in their attachment to tissue culture cells (50), vaginal epithelial cells (27), fallopian tube epithelium (74), and buccal epithelial cells (36).

These studies led to the view that the role of gonococcal pili in virulence was associated with their ability to promote adherence to the mucosal surfaces of the genital tract, a view which was strengthened by the observation that isolated pili readily attached to epithelial cells (4). Results of experiments in which gonococci were chemically modified to alter their surface charge suggest that pili participate in the first stage of a two-stage attachment process (16). Initially, pili are able to overcome the electrostatic barrier which exists between the negatively charged surfaces of the gonococcus and the host cell. This increases the probability of a closer approach, leading to a stable adhesion which involves other components of the gonococcal surface, including outer membrane protein PII (23), and may occur even when the bacterial cell is nonpiliated. The precise mechanism by which pili are able to overcome the electrostatic barrier is unclear; one possibility is that because of their small surface area, pili are less sensitive to the electrostatic repulsive forces than the surface of the bacterium is.

Fresh isolates of meningococci are also invariably piliated, but the role of pili in pathogenesis has been less extensively studied than that of gonococcal pili, and consideration of their interaction with host cells is complicated by the additional presence of a hydrophilic capsule on the surface of the bacteria. Nevertheless, pili do mediate adhesion of meningococci to nasopharyngeal cells and may therefore be important factors in establishing the carrier state (48). Isolates cultured from the blood and cerebrospinal fluid of patients with meningococcal disease are also piliated (9, 48, 49), and pili have been directly demonstrated in the cerebrospinal fluid of a child with meningococcal meningitis (47). Whether pili play any role in the ability of meningococci to transgress the blood-brain barrier or interact with meningeal tissues remains unclear.

Nature of the Pilus-Host Cell Interaction

Host cell receptors. Despite the established role of pili in adhesion to epithelial cells, the molecular basis of this interaction remains unclear, although considerable specificity is seen in in vitro experiments with respect to both anatomical location and the species from which the tissue is isolated. Thus, piliated gonococci adhere to isolated fallopian tubes from humans and higher primates but not to equivalent tissues from other animals (18). Purified gonococcal pili attach at a much higher density to cervical-vaginal and buccal epithelial cells than to erythrocytes, leukocytes, or fibroblasts (35). Similarly, piliated meningococci attach in much greater numbers to human nasopharyngeal and buccal epithelial cells than they do to anterior nasal cells (48).

Although the specificity of attachment with regard to both species and cell type tends to suggest recognition by pili of specific ligands on the epithelial cell surface, only limited evidence is available about the nature of such an epithelial cell receptor. Buchanan et al., using purified pili, reported that adhesion was inhibited by the addition of a number of different purified gangliosides or by pretreatment of buccal epithelial cells with exoglycosidases (5). They suggested that the human cell receptor was likely to resemble a ganglioside in structure. A similar conclusion was reached in studies with oligosaccharide-deficient clones of Chinese hamster ovary cells (14). In contrast, Trust et al. (66) reported that gangliosides had little effect on the adhesion of Pil$^+$ gonococci to buccal epithelial cells. However, pretreatment of the buccal cells either with sodium periodate or with a mixture of neuraminidase and glycosidase did reduce the attachment of Pil$^+$ gonococci to the level seen with a Pil$^-$ variant. Thus, the consensus of opinion implicates carbohydrate moieties present on the surface of epithelial cells as potential gonococcal pilus receptors, but their precise identity remains far from clear.

Pilus receptor-binding domain. The nature of the region(s) on the pilin molecule involved in recognition of host cells is also far from clear. Gubish et al. reported that the CNBr-2 fragment, obtained from pilin treated with cyanogen bromide, bound to Chinese hamster ovary cells in similar amount to intact pili, whereas the carboxy-terminal CNBr-3 did not attach (14). Adhesion was reduced by periodate or galactosidase treatment, suggesting that sugars present on the pilin were required for optimal attachment (14). Schoolnik et al., using erythrocytes, also reported that CNBr-2 (residues 8 to 84) had adhesive properties (43). In subsequent studies they reported that a tryptic fragment encompassing residues 31 to 111 bound to human endocervical cells but not to buccal epithelial cells or HeLa cells and postulated that this region encompassed the receptor-binding domain (42).

Since the studies described above and in the previous section were carried out with different strains and different cell types, it is perhaps not surprising that the nature of the pilus-host cell interaction has not been unambiguously defined. Additional complexity in interpretation occurs because the majority of the studies were carried out before the extent of potential structural and antigenic variation of pili was appreciated. Such variations may have an important influence on the interaction of pili with a variety of different cell types (see below).

Other Possible Pilus Functions

Interaction with PMN. The interaction of Neisseria species with polymorphonuclear leukocytes (PMN) has important consequences for the potential outcome of an infection. Several early studies showed that Pil$^+$ gonococci were more resistant to phagocytosis than were the equivalent Pil$^-$ variants (8, 10, 12, 58), suggesting an important additional

role of pili in virulence. Swanson et al., however, suggested that pili had only a minor role in gonococcus-PMN interactions and that an additional outer membrane component termed leukocyte association factor dominated the interaction (56, 57). Subsequently, leukocyte association factor was recognized to have the biochemical properties characteristic of outer membrane protein PII (53), and it was demonstrated that Pil$^-$ PII$^-$ variants showed considerably less interaction with PMN than did their Pil$^-$ PII$^+$ equivalents (24, 37).

Since the original studies were carried out before knowledge of PII variation was available, the association between piliation and resistance to phagocytosis was in some doubt, since changes in PII expression may have accompanied those in pilus expression. We therefore used a panel of variants of strain P9 with defined differences in pilus and/or PII expression in a chemiluminescence assay to determine initial interactions and in a phagocytic killing assay to determine the ultimate fate of the gonococci (72). In this study, leukocyte interaction was synonymous with possession of PII. When pairs of variants expressing the same PII but either Pil$^+$ or Pil$^-$ were compared, the chemiluminescence response was determined in each case by the particular molecular species of PII present, with pili having a negligible effect. Moreover, pili did not inhibit either uptake or intracellular killing, confirming the predominant role of PII in PMN interactions and suggesting that pili have little effect in resistance to phagocytosis by PMN.

Transformation. Gonococci are naturally competent for transformation throughout their growth cycle, with piliated cells being transformed at much higher frequencies than nonpiliated cells (45), although the mechanism by which pili enhance transformation is not known. Recently it was suggested that since gonococci readily undergo autolysis, they are therefore constantly exposed to their own deoxyribonucleic acid, leading to transformation events which may play a critical role in antigenic variation in pilus expression (44).

VARIATION IN PILUS EXPRESSION

Phase Variation

The original observations which led to the identification of pili on gonococci are the result of expression being subject to phase variation (2), so that although pili are lost on repeated subculture, Pil$^-$ cells may revert to the Pil$^+$ phase. Genetic analysis of phase variation has revealed complex mechanisms of piliation control, with two classes of Pil$^-$ variants (52). Members of one group are unable to revert to pilus production, whereas members of the other group revert at high frequency. The possible role of the Pil$^-$ phase in infection is unclear, but it appears likely that the reduced attachment associated with loss of piliation may allow the gonococci to leave the site of initial colonization and gain access to other locations. Similar considerations also apply to the spread of meningococci from the nasopharynx into the blood and cerebrospinal fluid. It has also been suggested that a nonadherent interim state could enhance gonococcal transmission, with reversion to the Pil$^+$ phase allowing subsequent adhesion to the mucosal surfaces of the infected individual (52).

Antigenic Variation

Considerations of the role of pili in pathogenesis are dominated by their extreme structural and antigenic diversity. Early studies revealed that gonococcal pili are immunogenic for laboratory animals (3) and that patients with gonorrhea develop anti-pilus antibodies (6). Despite their apparent considerable structural homology, based on amino acid analysis and peptide mapping, pili from different strains were found to be antigenically distinct. Antisera to purified pili raised in rabbits showed only limited cross-reactivity with pili from other strains, the amount of shared antigenicity between pili from heterologous strains usually being less than 10% (2).

Even greater antigenic diversity is generated by the fact that pili produced by a single strain undergo antigenic variation. Lambden et al. (25) purified pili from colonial opacity variants of strain P9 and found that pili from the transparent type had pilin with a subunit molecular weight of 19,500 (α-pili), whereas those from an opaque type had a molecular weight of 20,500 (β-pili). Another study with a number of strains showed that pilin M_r usually, although not always, varied between pairs of opacity variants (41). These observations suggested that alterations in subunit M_r might be linked to opacity and hence PII expression. However, further studies with strain P9 revealed two further pilus types (γ and δ) and showed that their expression was not linked to PII expression (22). This conclusion was confirmed by a study of other strains, which showed that a single strain could produce at least a dozen different pilus types (51). Antisera raised against variant pili produced by a single strain display only limited antigenic cross-reactivity (68). Recent studies of the genetic mechanisms of pilus variation show that the potential repertoire of pili expressed by a single strain may be even greater than indicated above (30).

Antigenic Shift during Gonococcal Infection

The possible occurrence of antigenic variation during natural infection was less easily established because of the ethical need for prompt antibiotic therapy, but was confirmed by comparing isolates taken from different sites in sexual partners. Gonococci were cultured from the urethras of male patients and from the urethras and cervices of their female partners, and surface antigen preparations were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11). Within each group of patients, the auxotype and the serotype of the outer membrane protein PI were identical, confirming their clonal derivation, but considerable variations were seen in expression of both pili and outer membrane protein PII. For example, one strain expressed pili of M_r 17,500 in the urethra of a male, 18,500 plus 19,000 in the urethra of a female partner, 17,000 in her cervix, and 18,300 in the cervix of a second female contact (75). Indeed, the M_r of pili differed between the isolates from the cervices and urethras of all female patients examined, suggesting that antigenic shift occurs commonly during the course of gonococcal infection. This view was confirmed by observations of isolates obtained during an outbreak caused by a penicillin-resistant gonococcus strain (46). In subsequent studies, human volunteers were subjected to urethral challenge and the pilins of the gonococci isolated during the resulting infection were analyzed (55). All reisolates were found to express pili which were structurally and antigenically distinct from those expressed by the input gonococci. The occurrence of extensive variation in each of the reported studies suggests that antigenic shift in pilus expression occurs commonly during the course of the natural infection and must play an important role in pathogenesis of gonococcal disease.

Antigenic Shift during Meningococcal Infection

Laboratory investigation of possible meningococcal antigenic variation is hampered by the fact that the changes in colony morphology which led to the discovery of antigenic variation in gonococci are not readily observed with meningococci. Nevertheless, variations in pilus M_r were detected following nonselective laboratory subculture of one strain (33). The occurrence of antigenic shift during meningococcal infection has been investigated by comparison of paired isolates obtained from the blood, cerebrospinal fluid, and nasopharynges of patients (59). Isolates from any individual produced identical deoxyribonucleic acid fingerprints and showed stability in expression of the class 2/3 and H.8 antigens, confirming their origin as a single strain. Variation in pilus expression was detected not only in strains expressing pili which contained the conserved gonococcal epitope recognized by monoclonal antibody SM1, but also in a strain which expressed non-SM1-reacting pili. Subsequent studies have revealed extensive variation in the M_r of SM1-reactive pilins produced by strains isolated from symptomatic and asymptomatic infected individuals during an epidemic of serogroup A meningitis, confirming the likely widespread occurrence of antigenic shift (1). Since meningococci produce one of two distinct classes of pili, both of which can undergo antigenic shift during infection, their antigenic repertoire appears to be equal to or even greater than that of gonococci.

Role of Antigenic Shift in Pathogenesis

Genetic studies (30) show that pathogenic *Neisseria* species have evolved with a significant proportion of their genome devoted to complex genetic mechanisms designed to ensure a continual change in the antigenic nature of the pili which they express. This, combined with the widespread occurrence of antigenic shift during natural infection, suggests that it must confer significant survival advantages on the bacteria, presumably allowing adaptation to a changing external environment. Clearly, one potential benefit would be expected to occur in interactions with the host immune system. Many studies have shown the potential protective effect of antibodies directed against gonococcal pili (see below). Thus, the ability to switch to expression of antigenically distinct pili would allow the gonococci to evade the effect of antibodies directed against the original colonizing variants. The effect would also be enhanced by a concomitant switch in the nature of the PII expressed (75). Although the role of anti-pilus antibodies in meningococcal disease is unclear, similar considerations may also apply and could, for example, explain the persistence of nasopharyngeal carriage despite the presence of the host immune response (13).

An additional potential effect of antigenic variation may influence the ability of both gonococci and meningococci to colonize host cells. Purified α- and β-pili from gonococcal strain P9 show striking differences in their ability to adhere to buccal epithelial cells, with α-pili showing a much greater affinity (25). This advantage was lost when the buccal cells were treated with glycosidases, and it was suggested that the α-pili recognize an oligosaccharide present on the surface of buccal cells but that β-pili do not (66). In contrast, when the piliated variants were compared for their ability to attach to and invade Chang conjunctival cells growing in tissue culture, the β-piliated variant showed much greater adhesion and hence virulence than did the α-piliated variant (68). The altered specificity for different cell types in vitro suggests that in vivo variation might endow the gonococci with the ability to colonize a variety of cell types found at different anatomical locations. Similar factors may well operate during meningococcal infection. Differences between the abilities of different strains to adhere to bucal epithelial cells and erythrocytes have been associated with differences in the mechanism of attachment of the pili which they express (65). The ability to interact specifically with different cell types may well be important in the pathogenesis of meningococcal disease, since the varied symptoms of infection result from a complex and poorly understood series of interactions between the bacteria and a variety of host cells.

STRUCTURAL AND IMMUNOCHEMICAL BASIS OF ANTIGENIC VARIATION

Considerable information has now accumulated from sequencing pilus structural genes from variants of a number of different strains expressing a variety of distinct pili. Variants have been isolated by colony selection (15, 32) and following natural infection (15) and experimental infection of both animals (32) and human volunteers (55). A clear model of the structural basis of pilus variation has emerged (Fig. 1). Pilins can be considered to contain three major regions, a region encompassing approximately the first 53 amino acids, which is highly conserved between pilins, a semivariable region (residues ca. 54 to 114), and a hypervariable region at the carboxy terminus. Structural variations in the semivariable region arise from amino acid substitutions, but in the hypervariable region insertions and deletions of up to four amino acids occur. Within the hypervariable region, two conserved sequences occur centered around the two cysteine residues (residues ca. 121 and 154) which form the disulfide bridge and loop; this may result from conservation of sequences at the deoxyribonucleic acid level that are involved in the genetic mechanisms of pilus variation (30). Sequence analysis of the cloned gene for expression of the SM1-reactive class of pilin from one meningococcal strain also conforms to this model (35a).

Thus, despite distinct antigenic specificity, variant pilins show a considerable degree of structural homology. Comparison of published variant pilin sequences from gonococcal strains MS11 (29) and P9 (32) and the SM1-reactive pilin from meningococcal strain C311 (35a) shows that they have approximately 80% of their amino acid residues in common. Variation in approximately 20% of the pilin must generate the extensive antigenic diversity seen, with conserved regions apparently being immunorecessive. In one series of studies, synthetic peptides were synthesized corresponding to a series of regions of one MS11 pilin. Immunization with intact pili was found to produce antibodies directed predominantly against peptides equivalent to residues 121 to 134 and 135 to 151, corresponding to the hypervariable region within the disulfide loop (39). Low levels of antibodies were directed against a weakly immunogenic determinant between residues 48 and 60. The authors suggested that pili had evolved so that the most immunogenic domain, the disulfide loop, was located on the surface of the pilin molecule and that amino acid substitutions could occur in this region, altering antigenic specificity without disrupting regions critical for pilus function.

The immunochemistry of intrastrain antigenic variation has been investigated by using monoclonal antibodies raised against variant pili of strain P9 (69, 73). The immunodominance of type-specific epitopes was confirmed in that from over 200 antibodies screened, only 1 monoclonal antibody

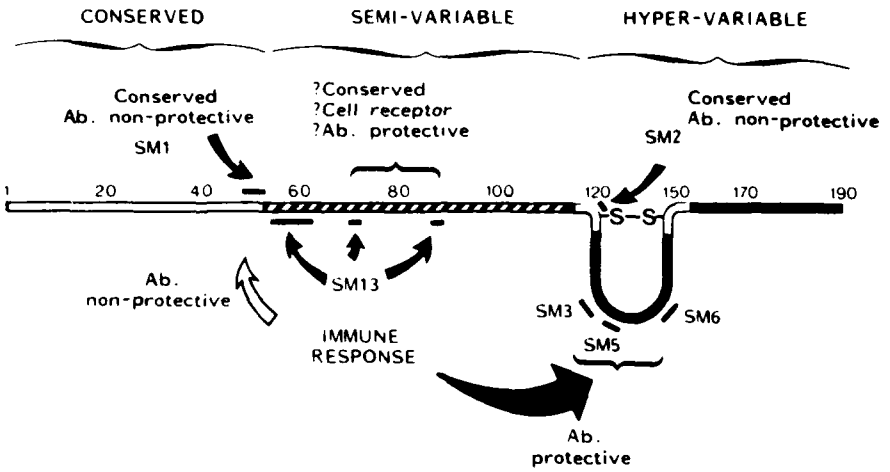

FIG. 1. Schematic diagram of the pilin molecule produced by gonococci and many strains of meningococci, showing conserved (□), semivariable (▨), and hypervariable (■) regions. The normal immune response is directed primarily against epitopes in the hypervariable region (➧), although low levels of antibodies (Ab.) are directed against conserved determinants (▷). Epitopes recognized by particular monoclonal antibodies are denoted (——), and with their potential protective effect is given.

(SM1) was obtained which reacted equally well with all gonococci tested, whereas a second (SM2) reacted with all of them, but to variable extents. The type-specific antibodies showed limited and variable reactivity with other strains. Four antibodies that all reacted with α-pili were tested against the pili expressed by eight variants from three groups of clinically related isolates. Only one isolate reacted, and then with a single antibody, suggesting that the antigenic specificity of a pilus is due to a particular combination of different epitopes, some of which may be independently shared with individual variants of other strains.

Comparison of the predicted amino acid sequence of cloned pilin genes with the immunological reactivity of the encoded pilins has allowed the amino acid sequence to be correlated with monoclonal antibody reactivity (32). The epitopes for three type-specific antibodies were found to depend on the presence of specific sequences in discrete regions within the disulfide loop, between residues 127 and 140. Each putative epitope was located in a hydrophilic domain with high β-turn probability. Reactivity with one type-specific antibody, however, was associated with the presence of three well-separated sequences (residues 56 to 63, 69 to 71, and 92 to 95). Each of the three domains had high β-turn potential, suggesting that the epitope was formed on the surface of the pilin molecule from the combination of three discontinuous regions all occurring within the semivariable region (Fig. 1).

The localization of each of the putative type-specific epitopes in hydrophilic domains with high turn potential would be in accord with their exposure and hence their immunogenicity. In contrast, the weakly immunogenic conserved epitopes recognized by antibodies SM1 and SM2, which have been identified by using synthetic peptides (J. E. Heckels and M. Virji, unpublished observations) (Fig. 1), are located between residues 49 and 53 and residues 118 and 127, respectively, in regions of moderate hydrophilicity but low turn potential.

PROSPECTS FOR A PILUS VACCINE

Biological Role of Anti-Pilus Antibodies

The association of pili with virulence has prompted many studies to determine the potential of pili for vaccination against gonorrhea. Antisera raised in laboratory animals by immunization with purified or partially purified pili have been shown to have a protective effect in a variety of biological systems. Anti-pilus antisera reduce the adhesion of both piliated gonococci (60) and purified pili (35) to human buccal epithelial cells. Antibodies to pili opsonize pili for phagocytosis by PMN (36) and macrophages (19) and protect tissue culture cells from the cytotoxic effect of challenge with gonococci (67). Anti-pilus monoclonal antibodies also inhibit adhesion, opsonize, and protect against infection (70, 71), and immunization with pili can also protect guinea pigs against infection following gonococcal challenge of subcutaneously implanted plastic chambers (24). However, in many of the above studies, the test and immunizing strains were identical, and little or no protection was observed with heterologous strains.

Anti-pilus antibodies can be detected in genital secretions from patients with gonorrhea, and such antibodies inhibit the attachment of the infecting strain to buccal epithelial cells (61, 63). Human volunteers immunized with pili produce detectable anti-pilus antibodies in both serum and genital secretions; these antibodies are also able to inhibit epithelial cell attachment (28, 64). Challenge of immunized volunteers with the homologous strain has shown statistically significant protection, as revealed by the size of the dose required to produce subsequent infection (2). These studies also suggested that the human immune response to pili might be less type specific than that seen in laboratory animals and so provide useful protection against infection. However, in large-scale field trial with male volunteers, no difference in protective effect was seen between the vaccine and placebo groups (62). Vaccinated volunteers developed gonorrhea despite high levels of serum antibody directed against their infecting strain. Thus, it seems likely that the levels of antibody present in genital secretions either were insufficient or perhaps reflect an immune response directed against a conserved but nonprotective epitope (see below).

The Problem of Antigenic Shift

One possible strategy to overcome the problems posed by the antigenic variability of pili is to design vaccination regimens to boost the immune response to conserved rather than type-specific epitopes. The protective effect of anti-

pilus antibodies directed against different pilus epitopes has been investigated by using a panel of monoclonal antibodies raised against pili from strain P9. The binding of ^{125}I-labeled variant pili to buccal epithelial cells was inhibited by the appropriate type-specific antibodies but not by the cross-reacting antibodies SM1 and SM2 (70), and the virulence of variants expressing different pili was also inhibited by the type-specific but not by the cross-reacting antibodies. Similarly, the type-specific antibodies promoted opsonization and killing by PMN, but the two cross-reacting antibodies had little effect (71). The cross-reacting antibody SM1 and one of the type-specific antibodies had the same isotype and bound to native pili in similar numbers and with similar avidity, demonstrating that the difference in biological activity was directly related to the nature of the epitope recognized. Antibody SM1 recognizes an amino acid sequence within the weakly immunogenic region encompassed by amino acid residues 48 to 60 (see above). Thus, it appears that even the low levels of cross-reacting antibodies which are seen on immunization with intact pili are directed against nonprotective antigenic determinants.

An alternative strategy to immunization with intact pili has been suggested by Rothbard et al., who reported that antibodies raised against the conserved CNBr-2 fragment produced antibodies which were more cross-reactive than those raised against intact pili and reacted with a peptide corresponding to residues 69 to 84 rather than 48 to 60 (39). Synthetic peptides corresponding to one variant pilus of strain MS11 were conjugated to carrier proteins and used to produce specific anti-peptide antibodies. The antibodies were tested for their ability to inhibit the adhesion to a human endometrial carcinoma cell line of a variant of the heterologous strain F62. Antibodies directed against residues 48 to 50 and 69 to 84 showed significant inhibition of attachment when used at high concentration, whereas antibodies directed against residues 48 to 60 were without effect (40). The authors suggested that synthetic peptides could be used to direct the immune response to normally nonimmunogenic epitopes, and since they could elicit cross-reacting, receptor-blocking antibodies, the peptides were promising candidate immunogens for the prevention of gonorrhea. The studies, however, showed a protective effect with a single heterologous strain and did not explore the possible consequences of antigenic variation within a strain. In subsequent experimental infections of human volunteers, Swanson et al. have demonstrated that significant antigenic variations in strain MS11 variants can occur at residues 69 to 84 and that these destroy the epitope recognized by a monoclonal antibody that inhibits epithelial-cell adhesion (55). They suggested that these data indicate that a peptide of residues 69 to 84 would not be effective as a gonorrhea vaccine. Comparative analysis of published sequences (15, 32, 55) does, however, suggest that a central portion of the peptide, residues 73 to 78, may be more highly conserved; unfortunately, it is not known whether antibodies directed against this region would have any inhibitory effect.

CONCLUSIONS

Pili appear to play an important role in the pathogenesis of gonococcal infections, and presumably also meningococcal infections, through their ability to promote adhesion to epithelial-cell surfaces. Although the molecular basis of the pilus-host cell interaction remains to be fully defined, the attraction of inducing anti-pilus antibodies to prevent initial colonization of mucosal surfaces remains. However, the potential use of pili for vaccination against *Neisseria* infections has been frustrated by their antigenic heterogeneity, since they have evolved so that the main immune response is directed against variable determinants and even the low levels of cross-reacting antibodies produced are directed against nonprotective epitopes. It remains to be seen whether alternative strategies involving selected fragments of the pilin molecule will enable the stimulation of an effective immune response which would protect against the almost limitless number of distinct antigenic types that appear possible.

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Gonococcal Vaccines

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The quest for a vaccine against *Neisseria gonorrhoeae* has been long, arduous, and, to date, unsuccessful. Indeed, some experts, citing the recurrent nature of gonococcal infections in some people, have believed the task to be impossible (2).

However, there has been at least one human challenge study that has demonstrated protection against the homologous infecting organism after immunization with purified gonococcal pili (4). Furthermore, *N. gonorrhoeae* is quite antigenic for humans, local and systemic immune responses have been demonstrated against virtually every gonococcal antigen studied, and relative resistance to infection has been correlated with a history of previous infections (22).

PATHOGENESIS

An understanding of the basis for a vaccine requires a working knowledge of the pathogenesis of the infection. The pathogenesis of a gonococcal infection can be broken down into five stages: (i) distant attachment, mediated by pili and perhaps pilus-associated proteins; (ii) close attachment, mediated primarily by cell wall protein antigens and perhaps lipooligosaccharides (LOS); (iii) ingestion by mucus secretory cells, which is mediated at least in part by protein I; (iv) transportation through the cell body in phagosomes, a host cell function; and (v) egestion through the basement membrane (although the proof for this last step is not absolute).

A variety of different immunological tests have repeatedly demonstrated the following: (i) a human antibody response is invoked by a gonococcal infection; (ii) the magnitude, antibody isotype, and antibody specificity of the response are unpredictable but tend to be more pronounced in women; (iii) there is a significant amount of cross-reactivity with antibody induced by other organisms; and, most importantly, (iv) to date, no correlation of the type or level of antibody has been made with protection. Local antibody, which functions primarily by blocking attachment of gonococci to eucaryotic cells, is present but at a reduced level (50).

ANIMAL MODEL AND IN VITRO CORRELATES WITH IMMUNITY

There is no animal model that correlates with the human infection. Thus, meaningful infection can be carried out only in the natural host, the human.

Without a relevant animal model, an in vitro correlate of immunity could serve as a guide (e.g., serum bactericidal antibodies served as the relevant in vitro correlate for the development of the successful meningococcal vaccine). Unfortunately, an in vitro correlate of human immunity has not yet been found. Thus, experimental studies in human volunteers and field trials must be relied upon if we are to fully understand the pathogenesis of gonococcal infections and to test the utility of vaccine candidates.

HUMAN VACCINE CHALLENGE STUDIES AND TRIALS

A number of vaccines have been studied in the past. In this brief review, however, only the most recent vaccine preparations will be discussed.

On the basis of (i) the demonstration that piliated gonococci are the most pathogenic for humans (17, 18), (ii) the successful human challenge study with a gonococcal pilus vaccine derived from the challenge organism (4), (iii) the demonstration of a consistent immune response following immunization (51), including the production of local antibody (24), and (iv) the suggestion that the pilus vaccine preparation might be broadly cross-reactive (51), a large gonococcal pilus vaccine trial involving 3,250 volunteers was undertaken in 1983. No overall protection was detected, although a significant proportion of the volunteers developed an antibody response (49). Therefore, a gonococcal pilus vaccine made up of the entire pilus derived by mechanical shearing and then purified by physico chemical means is unlikely as a potential vaccine candidate.

A protein I vaccine challenge study has also been conducted (E. W. Hook III, personal communication). The vaccine derived by differential centrifugation of disrupted gonococci was more than 85% pure for protein I. It was well tolerated, a significant antibody response was elicited, but it afforded no protection against an intraurethral challenge in men with the homologous organism.

Protection of volunteers after vaccination with Formalin-killed whole piliated organisms has also proved unsuccessful. All of the above vaccines were given parenterally.

POTENTIAL VACCINE CANDIDATES

Pili

Since the human challenge experiments of Kellogg et al. (17, 18), Brinton et al. (4), and Boslego et al. (J. Boslego, J. Ciak, P. Hitchcock, J. Swanson, E. C. Tramont, J. Sadoff, and J. Koomey, unpublished data) indicate a primary role for pili in the pathogenesis of gonorrhea, this review will discuss gonococcal pili in greater detail. Pili are extracellular hairlike structures that either radiate from or encase the gonococcal organisms (43, 47). Pili may allow the organism to attach to epithelial cells or may be antiphagocytic (6, 46, 48, 57). However, data for the latter are controversial (30). Pili are composed of identical pilin subunits with molecular weights of 15,000 to 22,000 (4, 33, 37). Pilins may contain receptor-binding domains (35, 38), although putative pilus-associated proteins may also have functional properties (19, 28, 41). Uropathogenic *Escherichia coli* cells have pili that are composed of pilin and pilus-associated proteins, one of which is the adhesin responsible for the attachment of the organisms to the urogenital tract (21). By analogy, one or more of the gonococcal pilus-associated proteins may also be important in the pathogenesis of gonococcal disease. Thus, the pilus-associated proteins may be future vaccine candidates.

Piliation is a variable state associated with an intact expression site in the gonococcal genome (13, 26, 42, 44). Phase variation from piliation to nonpiliation may involve a deletion event at the expression site (13) or give conversion, resulting in the expression of a missense pilin which cannot assemble (39, 42, 44). In addition, the changes in sequence at the expression site can result in the change of one pilin serotype to another (13, 26, 44). The pilin molecule is about 160 amino acids long. The first 53 amino-terminal amino acids are conserved (39). However, the rest of the molecule is marked by variability (13). Extreme variability occurs between two cysteines at positions 121 and 151 of the molecule. In addition to nucleotide changes which may result in amino acid substitutions, there may be deletions or insertions of enough deoxyribonucleic acid in the genome to delete or add several amino acids. The serological specificity of the response to pilus immunization in laboratory animals appears to be type specific (35, 37, 38). It also appears that in humans, type-specific antibody is protective (4). Because of this extreme variability, pilin immunization may not be feasible. The lack of protection in a field trial with a single pilus vaccine is consistent with this concept.

There do appear to be other, shorter sequences throughout the molecule which are frequently conserved (13, 53). Monoclonal and polyclonal anti-peptide antibodies to several areas in the molecule appear to be cross-reactive (35, 38, 53), and relatively less variability between positions 7 and 92 of the molecule has been most consistently found. This part of the molecule also appears to be immunorecessive in laboratory animals. Peptides made from this amino acid sequence (residues 69 to 84 and 41 to 50) generate cross-reactive anti-pilus antibody that blocks the attachment of gonococci to human cervical cell culture lines (35, 37, 38). It is noteworthy that in the human field trial of the pilus vaccine, cross-reactive antibody arose as a secondary or anamnestic response, indicating the preexistence of anti-pilus antibody. By Western immunoblot analysis, this antibody appeared to be directed to epitopes located in all areas of pilin (unpublished data). Other studies have shown the preexistence of antibody to several outer membrane antigens and have proposed that meningococcal or other bacterial carriage may be responsible for these antibodies (15).

Pilus-associated proteins have been reported by several investigators (19, 28, 41). One study found that there were at least 26 different proteins produced by a piliated organism that were not present in its nonpiliated counterpart (19). Another study found that antibody generated to pili purified by deoxycholate-urea buffer resulted in recognition of not only pili but also five other proteins that were present in piliated organisms but not in nonpiliated organisms (28). Based on binding of both piliated and nonpiliated gonococci to glycolipids, it is postulated that a protein present on both cell phenotypes binds to several glycolipids found in human cervical cell lines (lactosylceramide and gangliotriaosylceramide) (41). The degree of variability of these proteins has not yet been fully ascertained, although they are unlikely to be as variable as the pilin molecule, since they are present in small quantities and may generate little if any immune response in a natural infection. The immunologic pressure for them to vary would therefore be small. Thus, these proteins may be excellent future vaccine candidates.

Protein I

Protein I is the major outer membrane protein of *N. gonorrhoeae*. It is a porin and therefore is responsible for the entry of small molecules through the gonococcal outer membrane (3). Although there is interstrain variation, there is no intrastrain variation. There are two structurally different proteins I, IA and IB, and each has several serotypes (3). Release of protein I into the pericellular area may result in the endocytosis of gonococci by the host cell (3). Antibodies to protein I are bactericidal, and the protein I type may be associated with serum resistance (16, 52, 54). The nature of this association is discussed more completely elsewhere in this issue (31).

Recurrent salpingitis was not associated with isolates processing the same protein I serotype as the isolate from the initial episode of salpingitis (zero of nine patients). On the other hand, cervicitis occurring after an episode of salpingitis was associated with strains possessing the same protein I serotype in 5 of 10 patients. The implication is that the immune response to protein I during salpingitis may result in protein I serotype-specific protection against recurrent gonococcal salpingitis (5).

Protein I is largely embedded in the gonococcal membrane. Some areas are exposed, as determined by enzymatic digests of the outer membrane and genetic experiments involving the construction of hybrid porins (3, 7, 36, 45). Because of the small number of serotypes and the lack of intrastrain variation, protein I has been considered a vaccine candidate. However, a recent study has shown that not all organisms in a population of gonococci may have the protein I epitope(s) exposed on their surface (34). As mentioned above, a human vaccine trial with protein I did not protect against an intraurethral challenge with the homologous strain. This obviously does not rule out the possibility of protection against salpingitis.

Protein II

Protein II is a heat-modifiable protein responsible for the opacity of colonies grown on agar (3). It has been implicated in the adhesion of the organisms to epithelial cells, as well as adhesion between gonococci (3, 20). Progeny of a single gonococcus can produce several protein II types (39). Protein II vaccines would be restricted by the great variability of the antigen.

Protein III

Protein III has been found in all gonococcal strains. It appears not to be variable. Monoclonal antibodies to some epitopes appear to be bactericidal (55). However, recent studies have indicated that IgG present in normal human serum, which blocks bactericidal activity, is directed to protein III (32). Thus, protein III must be viewed with caution as a potential vaccine candidate. Perhaps protein III epitopes (e.g., peptides) that do not raise bactericidal blocking antibodies may prove effective as vaccines.

LOS

LOS, like many of the other antigens of gonococci, has been found to have great variability (12). Anti-LOS antibody is bactericidal (1, 11, 12, 23, 56). LOS may be responsible for the destruction of the host mucosa by acting as a toxin on the mucosal epithelial cells (11). Additionally, LOS determinants share homology with some blood group antigens (23). The homologous determinants may engender tolerance to the common epitopes of LOS or may serve as receptor-binding sites for the gonococci. A vaccine containing LOS

should be considered, but, like many of the other outer membrane antigens, this endeavor will be hindered by the variability of the antigen.

Major Iron-Regulated Protein

The major iron-regulated protein is a 37,000-molecular-weight protein that enables gonococci to utilize iron (25, 27). Following disseminated gonococcal disease, there is an antibody response to the major iron-regulated protein (10). Occasionally, there is a response following uncomplicated local infections (10). Since this protein may be responsible for the survival of the organism in humans, antibody directed to it may be protective. This protein is discussed in more detail elsewhere in this issue (9).

H.8 Antigen

H.8 is a common antigen found in the outer membrane of pathogenic *Neisseria* species. It is unusual in that it is proline and alanine rich and appears to be very hydrophobic (40). Following local infection, antibody to H.8 develops in some individuals (15). Like protein I, H.8 may not be equally exposed on all organisms of a population of gonococci, since electron micrographs show variability in the binding of gold-labeled anti-H.8 antibody (15). H.8 is discussed in more detail elsewhere in this issue (7).

Capsule

A gonococcal capsule associated with resistance to phagocytosis has been described but never isolated (14). Therefore, it is an unlikely vaccine candidate.

IgA Protease

Gonococci elaborate an IgA protease which cleaves IgA immunoglobulin, but its role in infection has not been clearly defined (29).

CONCLUSIONS

Despite much effort and many advances in molecular biology, a vaccine for *N. gonorrheoea* remains an elusive goal. The challenge is made greater by the lack of an animal model and the fact that an effective immune response has never been demonstrated. Piliation is an absolute requirement for urethral infection in men. A pilus vaccine protected men in a challenge study involving the use of a carefully selected clone representing the homologous strain from which the vaccine was made but failed to protect in a field trial. Nevertheless, gonococcal pili or pilus-related proteins remain attractive vaccine candidates. Protein I, protein II, protein III, the major iron-regulated protein, H.8, and LOS are also potential candidates. Indeed, one or more of these cell membrane antigens may be relatively more important in protecting against salpingitis, the complication of gonorrhea that results in the highest morbidity rate. Testing this hypothesis would be very difficult. Finally, it may be time to consider a different strategy, local vaginal immunization. Protecting one-half of the partnership in a sexually transmitted disease will protect the other!

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DNA Restriction and Modification Systems in *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae appears to be a genetically isolated species. There are no known bacteriophages capable of infecting this organism, and although most gonococcal strains carry plasmids, the variety of those plasmids is amazingly small. Only two plasmid species might be described as native to the gonococcus (1). The only other deoxyribonucleic acid (DNA) molecules that have ever been shown to enter this species are the penicillinase-producing plasmids (which probably originated in a *Haemophilus* species) (1), and the *tetM* determinant (which probably entered via *Mycoplasma* or *Ureaplasma* species) (10).

Despite this rigid barrier against the entry of foreign DNA, gonococci are highly competent for transformation (1), and it has been suggested that transformation may be the genetic system that results in the antigenic variation of several gonococcal surface components (9, 12). Thus, gonococci appear to have evolved a system that rigidly excludes foreign DNA, but still allows the free exchange of genetic information within the species. Exclusion of foreign DNA could be achieved by an efficient restriction barrier. Restriction barriers result from the production of restriction endonucleases which bind to, and cleave, a specific DNA sequence (17). Most restriction endonucleases will cleave unmodified DNA sequences only, and a cell that is producing a restriction enzyme will protect its own DNA from degradation by simultaneously producing a modification enzyme that binds to, and methylates, the DNA sequence recognized by the restriction enzyme (11).

Any variation, within the species, in these restriction modification systems might be expected to limit genetic exchange during mixed infections and perhaps some of the sequence diversity generated during antigenic variation (9). One such barrier to intraspecies genetic exchange has been directly demonstrated and seems to act specifically on DNA transferred by transformation, rather than conjugation (19, 20). This reflects the nature of the incoming DNA during these two genetic exchange processes. DNA is single stranded as it enters the recipient cell during conjugation and is therefore resistant to the action of most restriction enzymes. On the other hand, DNA fragments are taken up as double-stranded molecules during transformation of the gonococcus (1).

The true extent to which such barriers limit genetic exchange within this species was unknown, and the result has been an increased interest in characterizing both the number and the distribution of the gonococcal restriction modification systems.

REVISION OF NOMENCLATURE

Recently, an agreement was reached among the various laboratories involved in isolating restriction enzymes and methylases from *Neisseria gonorrhoeae* to revise the names of the gonococcal enzymes so that the nomenclature system properly reflects that used for all other bacterial species (17).

Restriction Endonucleases

The revised names for the previously characterized gonococcal restriction enzymes that had been misnamed are shown in Table 1. The new names contain a reference to the particular strain from which a restriction enzyme was isolated. An enzyme activity denoted R.*Ngo*X (4; M. K. Duff, M.Sc. thesis, Monash University, Clayton, Australia, 1986), has since been shown to consist of a mixture of two restriction enzymes, isoschizomers of R.*Hae*II and R.*Hph*I (4a).

Methylases

Korch et al., in an analysis of the DNA sequence of the gonococcal cryptic plasmid pJD1 from strain 82409/55, were able to obtain evidence that specific bases were methylated (5–7). The presence of these bases in palindromic sequences was used to suggest the presence of seven different type II methylases (6). A single incidence of a methylated base in a nonpalindromic sequence was suggested to be the result of the action of a type III methylase (7). Each of these putative methylases was given a specific name (6, 7); this has raised several nomenclature problems. No methylases have actually been characterized in this strain, and cases of single methylase enzymes with multiple sequence specificities have been reported (24). Again, no reference to the particular strain was made in the methylase names. The revised nomenclature (Table 2) has given names to the methylated DNA sequences; for example, the sequence methylated by the putative methylase M.*Ngo*I has been named S.*Ngo*I, where the S designates a methylase specificity, thus avoiding the issue of whether the enzyme that methylates this particular sequence can also methylate other sequences. The names for the putative methylases in strain 82409/55 have been discarded, and as gonococcal methylases (from this or any other strain) are characterized, they will be given names that indicate the strains from which they were isolated.

RESTRICTION ENZYMES

A number of restriction enzymes have been partially purified from *N. gonorrhoeae* and are listed in Table 3. Thus far, five different specificities have been identified, including a methylation-dependent endonuclease (4, 4a; Duff, M.Sc. thesis). All of the restriction enzymes so far identified are isoschizomers of characterized enzymes from other species (17).

METHYLASES

When DNA is directly extracted from a particular gonococcal strain, it is commonly resistant to cleavage by a variety of restriction endonucleases (4, 5, 13–15, 20, 22, 23, 25). Such cleavage resistance is undoubtedly the result of methylation of restriction enzyme recognition sites, but it is

TABLE 1. Revised nomenclature for gonococcal restriction enzymes

Previous name	Recognition sequence	Isochizomer	Strain	Revised name	Reference(s)
R.*Ngo*I	PuGCGCPy	R.*Hae*II	?[a]	R.*Ngo*WI	17
R.*Ngo*II	GGCC	R.*Hae*III	CDC66	R.*Ngo*CI	2, 3
R.*Ngo*III	CCGCGG	R.*Sac*II	KH7764-45	R.*Ngo*KI	13, 14
			JKD109	R.*Ngo*JI	4; Duff, M.Sc. thesis
			JKD211	R.*Ngo*DI	4; Duff, M.Sc. thesis
R.*Ngo*IX	G[m6]ATC[b]	R.*Dpn*I	JKD211	R.*Ngo*DIII	4, 4a; Duff, M.Sc. thesis
R.*Ngo*X	PuGCGCPy + GGTGA	R.*Hae*II + R.*Hph*I	JKD109	R.*Ngo*JII + R.*Ngo*JIII	4, 4a; Duff, M.Sc. thesis
			JKD211	R.*Ngo*DII + R.*Ngo*DIV	4, 4a; Duff, M.Sc. thesis

[a] Producing strain unknown.
[b] ^{m6}A, N^6-Methyladenine.

not possible to use such data to directly infer that a particular strain is producing a methylase with the same specificity as the restriction enzyme used in the assay. The sequence recognized by the methylase may merely be contained within, or overlap, the recognition site for the restriction enzyme. For example, DNA from most gonococcal strains is resistant to cleavage by the restriction enzyme *Not*I, which recognizes the sequence 5′-GCGGCCGC-3′ (17; J. K. Davies, unpublished data). Such cleavage resistance is almost certainly due to the presence of a methylase that recognizes part of the *Not*I sequence, i.e., 5′-GGCC-3′ (see below). Only when a particular strain has been demonstrated to produce an isoschizomer of the restriction enzyme used in the assay is it reasonably safe to infer that it is also producing a methylase with the same specificity. Thus, it is reasonable to assume that the strains listed in Table 3 are also producing methylases with specificities identical to those of the listed restriction enzymes.

Evidence for Methylase Activity

There have been direct demonstrations of methylase activity in extracts from gonococcal cells (16; Duff, M.Sc. thesis). Such extracts have been shown to possess both adenine and cytosine methylase activity (16).

Purified Methylase Activities

Only in a few cases has a methylase been purified to such an extent that a particular sequence specificity can be assigned to an individual enzyme. The enzymes that have been purified, and the sequences that they recognize, are listed in Table 4. Among these is a cytosine methylase, M.*Ngo*BI, with the specificity that Korch suggested might be the target for a type III methylase (7). M.*Ngo*BI, however, shows all the characteristics of a type II enzyme (15) and is part of one of the small group of type II restriction modification systems that recognize nonpalindromic sequences (17). The same strain also produces appreciable amounts of another methylase, M.*Ngo*BII, that methylates an interrupted nonpalindromic sequence (15). The existence of this methylase was entirely unexpected, since the strain concerned does not produce appreciable amounts of a restriction enzyme with the same recognition sequence, nor is there an available isoschizomer that might have been used in an assay for cleavage resistance.

CLONED GENES ENCODING RESTRICTION MODIFICATION SYSTEMS

The other way in which the presence of a restriction modification system can be directly demonstrated is through the cloning of the genes for the enzymes. One advantage of this direct approach (see below) has been the demonstration that such systems can be isolated from strains that do not produce appreciable amounts of the relevant methylase or restriction enzyme.

Cloning Procedures

The procedure used to clone the gonococcal genes is based on a general method that has been used to clone genes from a variety of restriction modification systems. The procedure aims to isolate a methylase gene, but because the relevant restriction enzyme gene is often closely linked, the genes for the entire restriction modification system are often found on the cloned DNA fragment. The method relies on the availability of an isoschizomer for the restriction enzyme concerned and the presence of at least one recognition site for that enzyme in the plasmid cloning vector used. A genomic library is constructed in the cloning vector in *Escherichia coli*, and this construction is followed by a bulk extraction of the recombinant plasmids. This mixture of recombinant plasmids is then digested with the isoschizomeric restriction enzyme and retransformed into *E. coli*. The only recombinant plasmids that should be resistant to cleavage, and therefore able to replicate in *E. coli*, are those that carry the relevant methylase gene.

Cloned Methylase and Restriction Enzyme Genes

The gonococcal restriction enzyme and methylase genes that have been cloned thus far are listed in Table 5. As might be expected, in one case the entire restriction modification system was cloned. It is also worth noting that this approach has been successful with strains that do not produce the relevant methylase in amounts sufficient to allow its purifi-

TABLE 2. Revised nomenclature for gonococcal methylase specificities

Putative methylase name[a]	Methylated sequence	Restriction enzyme acting at same site	Name of methylase specificity
M.*Ngo*I	PuGCGCPy	R.*Hae*II	S.*Ngo*I
M.*Ngo*II	GGCC	R.*Hae*III	S.*Ngo*II
M.*Ngo*III	CCGCGG	R.*Sac*II	S.*Ngo*III
M.*Ngo*IV	GCCGGC	R.*Nae*I	S.*Ngo*IV
M.*Ngo*V	GGNNCC	R.*Nla*IV	S.*Ngo*V
M.*Ngo*VI	GATC	R.*Mbo*I	S.*Ngo*VI
M.*Ngo*VII	GC(G/C)GC	None	S.*Ngo*VII
M.*Ngo*VIII	GGTGA	R.*Hph*I	S.*Ngo*VIII

[a] These names have now been discarded.

TABLE 3. Restriction enzymes isolated from *N. gonorrhoeae*

Specificity	Recognition sequence	Isoschizomer	Strain	Name	Reference(s)
S.*Ngo*I	PuGCGCPy	R.*Hae*II	?[a]	R.*Ngo*WI	17
			JKD109	R.*Ngo*JII	4, 4a; Duff, M.Sc. thesis
			JKD211	R.*Ngo*DII	4, 4a; Duff, M.Sc. thesis
S.*Ngo*II	GGCC	R.*Hae*III	CDC66	R.*Ngo*CI	2, 3
			P9	R.*Ngo*PII	21–23
			Pgh3-2	R.*Ngo*SI	19, 20
S.*Ngo*III	CCGCGG	R.*Sac*II	KH7764-45	R.*Ngo*KI	13, 14
			P9	R.*Ngo*PIII	21–23
			JKD109	R.*Ngo*JI	4; Duff, M.Sc. thesis
			JKD211	R.*Ngo*DI	4; Duff, M.Sc. thesis
S.*Ngo*VIII	GGTGA	R.*Hph*I	JKD109	R.*Ngo*JIII	4, 4a; Duff, M.Sc. thesis
			JKD211	R.*Ngo*DIV	4, 4a; Duff, M.Sc. thesis
			WR302	R.*Ngo*BI	15a, 19
—[b]	G[me]ATC[c]	R.*Dpn*I	JKD211	R.*Ngo*DIII	4, 4a; Duff, M.Sc. thesis

[a] Producing strain unknown.
[b] —, No specificity name.
[c] [me]A = N⁶-methyladenine.

cation (R. Chien, A. Piekarowicz, and D. Stein, unpublished data) and that a restriction enzyme gene can be cloned from strains that do not produce identifiable quantities of the enzyme (R. H. Chien, D. C. Stein, K. Floyd, M. So, and H. S. Seifert, unpublished data).

EVIDENCE FOR ADDITIONAL METHYLASE ACTIVITIES

There is evidence to suggest that gonococci possess additional, as yet uncharacterized methylases. First, Stein and co-workers have cloned the gene for a methylase with a specificity different from that of any of the known gonococcal enzymes (D. C. Stein, personal communication). Second, it has been known for some time that some strains of *N. gonorrhoeae* produce an adenine methylase (16; Duff, M.Sc. thesis). As yet, the gene for this methylase has not been cloned, nor has the methylase itself been purified. There is some evidence, although no direct proof, that the adenine methylase activity in these strains is the result of a single enzyme that recognizes the sequence 5'-GATC-3' (4, 4a, 13–15, 20, 25). There is also no report of a restriction enzyme that recognizes this sequence when it is unmethylated (an isoschizomer of R.*Mbo*I), although a restriction enzyme that recognizes the methylated sequence has been found in a strain that lacks adenine methylase activity (Table 3), i.e., an isoschizomer of R.*Dpn*I.

CHARACTERIZED RESTRICTION MODIFICATION SYSTEMS

A consolidated list of all the restriction enzymes and methylases that have been purified or cloned from *N. gonorrhoeae* is shown in Table 6. The list includes enzymes with eight different specificities and six of the eight specificities postulated by Korch et al. (6, 7). If M.*Ngo*BII (for which no restriction enzyme counterpart is known) is put to one side, the gonococci possess at least seven restriction modification systems.

TABLE 4. Purified gonococcal methylases

Specificity	Recognition sequence	Strain	Purified methylase	Reference(s)
S.*Ngo*II	GGCC	WR220	M.*Ngo*AI	15, 15a
S.*Ngo*VIII	GGTGA	MUG116	M.*Ngo*BI	15
—[a]	GTAN₅CTC	MUG116	M.*Ngo*BII	15

[a] —, No specificity name.

DISTRIBUTION OF RESTRICTION MODIFICATION SYSTEMS

Although the data in Table 6 indicate the number of restriction modification systems so far identified in the species, they do not indicate how many of these systems are usually possessed by an individual strain, and that is the information needed to ascertain whether restriction barriers might hinder the free exchange of genetic information within the species. The results cited above seem to indicate that when a gonococcal strain modifies its DNA so that it is resistant to cleavage by an isoschizomer of a characterized gonococcal restriction enzyme, that strain carries the methylase gene whether or not the methylase is being produced in amounts sufficient to be purified. In addition, it seems that such a strain also carries the gene encoding the corresponding restriction enzyme, whether or not the restriction enzyme is also being produced in amounts sufficient to be characterized. It has been suggested that this low level of production of some enzymes may be related to the growth phase of the cells (Chien, et al., unpublished). Some indication of the distribution of the known gonococcal restriction modification systems might therefore be gathered by looking at how often the DNA of individual strains is resistant to cleavage by isoschizomers of the known gonococcal restriction enzymes.

Incidence of Restriction Modification Systems

From the above criteria, and on the basis of our own surveys of different strains (M. K. Duff and J. K. Davies, unpublished data) and any published information, it seems that most gonococcal strains possess at least six of the restriction modification systems listed in Table 6. A small proportion of strains (perhaps 5%) seem to lack the S.*Ngo*II system, and an even smaller fraction lack the S.*Ngo*III system. The major variation, however, seems to be in the ability to produce the adenine methylase referred to above. We estimate that perhaps 30% of gonococcal strains have the ability to methylate the sequence 5'-GATC-3'.

TABLE 5. Cloned gonococcal restriction enzyme and methylase genes

Specificity	Recognition sequence	DNA resistant to cleavage with:	Strain	Enzyme(s) encoded on cloned DNA fragment	Reference(s)
S.*Ngo*I	PuGCGCPy	R.*Hae*II	P9	M.*Ngo*PI	23
S.*Ngo*II	GGCC	R.*Hae*III	P9	M.*Ngo*PII	23, 23
S.*Ngo*IV	GCCGGC	R.*Nae*I	MS11	R.*Ngo*MI, M.*Ngo*MI	R. H. Chien et al., unpublished
S.*Ngo*V	GGNNCC	R.*Nla*IV	MUG116	M.*Ngo*BIII	R. Chien et al., unpublished

Potential Barriers to Genetic Exchange

It would seem that the DNA from the few strains that lack the S.*Ngo*II and S.*Ngo*III systems would not be successfully transformed into the majority of gonococcal strains. Indeed, one of these restriction barriers has been directly demonstrated (19, 20). The main barrier to free genetic exchange within the species, however, seems to involve enzymes that act on the sequence 5′-GATC-3′.

ADENINE METHYLATION

As mentioned previously, many gonococcal strains appear to produce an adenine methylase that recognizes the sequence 5′-GATC-3′ (4, 4a, 13–15, 20, 25). Since no restriction enzyme recognizing the same sequence has ever been isolated from gonococci, it has been suggested that this methylase may be the equivalent of the *dam* methylase of *E. coli* (13, 14, 18, 25). The *E. coli* enzyme is not part of a restriction modification system but appears to have a variety of roles within the cell, including strand discrimination during mismatch repair (18). These characteristics do not seem to apply to the gonococcal enzyme. First, strains lacking this methylase do not appear to be hypermutable, as do the *dam* mutants of *E. coli* (13, 14). Second, a strain that lacks this methylase has been shown to be producing a restriction enzyme that cleaves the methylated sequence (4, 4a; Duff, M.Sc. thesis). This situation seems reminiscent of that found in *Streptococcus pneumoniae* (8).

Some strains of *S. pneumoniae* produce R.*Dpn*I, which cleaves the methylated sequence 5′-G^{me}ATC-3′, while others contain the complementary enzyme R.*Dpn*II, which cleaves only the unmethylated sequence 5′-GATC-3′ (8). Cells that produce R.*Dpn*I have no need to protect their DNA against this enzyme, whereas cells that produce R.*Dpn*II must produce a methylase to modify this site. It has been shown that the genes responsible for one restriction phenotype are not present in cells of the opposite phenotype but that each strain does share sequence homology on either side of the regions of the chromosome encoding the restriction enzymes (8). Therefore, the complementary restriction systems are found in nonhomologous and mutually exclusive cassettes, which are apparently inserted into a specific position in the chromosome. Whether or not such a system exists in the gonococcus remains to be demonstrated.

CONCLUSIONS

N. gonorrhoeae can efficiently exclude foreign DNA because of its multiple restriction barriers. This plethora of restriction modification systems does not limit the exchange of genetic information within the species, because most strains protect their DNA against cleavage by all of the gonococcal restriction enzymes. The only barrier to this free flow of genetic information seems to be the existence of a mutually exclusive set of restriction systems that recognize methylated or unmethylated versions of the sequence 5′-GATC-3′.

TABLE 6. Characterized gonococcal restriction modification systems

Specificity	Recognition sequence	Isoschizomer	Restriction enzyme	Methylase
S.*Ngo*I	PuGCGCPy	R.*Hae*II	R.*Ngo*WI R.*Ngo*JII R.*Ngo*DII	M.*Ngo*PI
S.*Ngo*II	GGCC	R.*Hae*III	R.*Ngo*CI R.*Ngo*PII R.*Ngo*SI	M.*Ngo*PII M.*Ngo*AI
S.*Ngo*III	CCGCGG	R.*Sac*II	R.*Ngo*KI R.*Ngo*PIII R.*Ngo*JI R.*Ngo*DI	Inferred, none characterized
S.*Ngo*IV	GCCGGC	R.*Nae*I	R.*Ngo*MI	M.*Ngo*MI
S.*Ngo*V	GGNNCC	R.*Nla*IV	None known	M.*Ngo*BIII
S.*Ngo*VIII	GGTGA	R.*Hph*I	R.*Ngo*JIII R.*Ngo*DIV R.*Ngo*BI	M.*Ngo*BI
—[a]	G^{me}ATC[b]	R.*Dpn*I	R.*Ngo*DIII	None
—[a]	GTAN$_4$CTC	None known	None known	M.*Ngo*BII

[a] —, No specificity name.
[b] ^{me}A = N^6-methyladenine.

There remains one important, and unanswered question. If the purpose of a restriction system is to exclude foreign DNA, this can be achieved by producing large amounts of a single restriction enzyme recognizing a 4-base-pair sequence. Why, then, have the gonococci maintained the ability to produce at least six restriction barriers? This is even more puzzling, considering that the gonococcal transformation system apparently has the ability to discriminate between gonococcal DNA and DNA from other sources, and to preferentially take up DNA from its own species (1). Why, then, the need for any restriction barrier?

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Interactions of *Neisseria gonorrhoeae* with Human Neutrophils

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The hallmark of acute gonococcal urethritis in males is the presence of gram-negative intracellular diplococci. Not only has this observation served as a diagnostic indication of gonorrhea, it has also suggested that *Neisseria gonorrhoeae* has the capacity to survive the massive inflammatory response of the host during infection. Thus, the interaction of gonococci with human phagocytic cells has both clinical and experimental significance.

It is the purpose of this review to discuss experiments performed during the past several years that deal with the interactions between gonococci and phagocytes. For the most part, this review will concentrate on polymorphonuclear leukocytes (PMNs), since their presence in purulent exudates is most remarkable and has been studied vis-à-vis *N. gonorrhoeae* to a greater extent than that of mononuclear phagocytes. Moreover, since gonococci are obligate human pathogens and since no cost-effective animal model mimics human infections, only experiments with human materials will be reviewed, except when comparisons help us to better understand the molecular events involved in gonococcus-PMN interactions.

Intraleukocytic killing of gonococci is the final event in the process of interest. However, killing requires certain sequential events which, at each step, can be resisted by gonococci. For instance, gonococci may resist phagocytes by killing them, inhibiting their chemotaxis, resisting ingestion, or inhibiting or surviving intraleukocytic killing mechanisms. Accordingly, this review is divided into three sections: extracellular events, cell surface events, and intracellular events. Although gonococci and other pathogenic bacteria are subjected to similar processes, research dealing with gonococci and PMNs has provided new information regarding phagocytosis, phagocytic killing, and the biology of gonococci. Therefore, we have sought to identify how studies dealing with gonococci and PMNs have helped to advance not only the field of gonococcal pathogenesis but also the biology of PMNs. Where there appear to be answers to certain questions, we have provided them. Where questions remain, we have pointed them out in a manner that we hope will spur new approaches to an old problem. Finally, as is often the case with gonococci, results and conclusions from many early experiments that examined the interaction of gonococci and PMNs must be reevaluated as the genetics, physiology, and structure of *N. gonorrhoeae* have become better understood.

EXTRACELLULAR EVENTS

In considering the extracellular events that take place during the initial phases of phagocytosis, three topics are important: chemotaxis, opsonization, and cytotoxicity. The first two favor the host, whereas the last (bacterium-mediated cytotoxicity) may help gonococci evade the initial defensive response of the host. Only a limited number of studies provide an indication of the events occurring prior to the physical interaction of gonococci with the surface of the phagocyte.

Chemotaxis

Chemotaxis is defined as the migration of phagocytic cells up a concentration gradient that often originates from infected areas. It was one of the first biological processes noted in immunological research and was central to the Metchnikoff (66) phagocytic theory of host defense. The capacity of PMNs to infiltrate tissues and fluids in response to chemotactic signals may shed light on the massive presence of PMNs in acute gonococcal urethritis. Conversely, down-regulation of the chemotactic process may help to explain asymptomatic infections. Thus, it is important to know the nature of the chemotactic signals recognized by PMNs during gonococcal infection and whether they differ during the course of acute, asymptomatic, or invasive forms of disease.

The principal chemotactic signal relevant to the migration of PMNs to gonococci is C5a, which is generated by cleavage of C5 by C5 convertase. This complement-derived neutrophil chemoattractant has been shown by Densen et al. (24) to be markedly increased in serum exposed to serum-sensitive as opposed to serum-resistant gonococci. This is important since serum-sensitive gonococci cause significantly greater inflammatory symptomology in women with acute salpingitis than the low-level symptomology seen in individuals with disseminated gonococcal infections that are typically caused by serum-resistant strains (87). Thus, the acute nature of salpingitis may be directly related to the capacity of serum-sensitive strains to increase the magnitude of C5a generation. This would stimulate PMN migration, leading to the severe inflammation seen in this form of gonorrhea.

Intact and sonicated gonococcal peptidoglycans (PG) activate the classical complement pathway in an antibody-dependent manner (79), thereby generating C5a. However, lysozyme digestion of sonicated PG abrogates this activity, but PG that is O acetylated is resistant to such digestion (90). Accordingly, PG fragments containing glycosidic linkages that are liberated in vivo and are recognized by antibody to PG should have a bearing on inflammation.

Cytotoxicity and Interference with PMN Functions

Microorganisms, through the production of leucocidins, have the ability to resist phagocytes by killing them prior to being ingested. Microscopic evidence suggests that some PMNs may fall victim to toxic substances released by gonococci. Visualization of purulent exudates reveals not only abundant numbers of intact PMNs but also phagocytes that appear to be damaged or disintegrating (18, 19, 68, 72). However, since the fully mature PMN has a half-life of about

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8 h, it is difficult to distinguish whether such PMNs have been attacked by gonococcal cytotoxic agents or are merely succumbing to old age. Certain gonococcal cell envelope components may participate directly in damaging PMNs. Gonococci, like other gram-negative bacteria, release outer membrane vesicles that may indirectly serve to deliver toxic compounds. Hence, potential cytotoxic agents released by gonococci might be individual factors released into the environment or outer membrane (OM) components released as part of membrane vesicles. Casey et al. (19) presented evidence that gonococci produce a cytotoxic substance with the ability to lyse PMNs. The substance did not appear to be lipopolysaccharide (LPS), which is known to damage fallopian tube tissue (21), or protein. No further reports regarding the nature or mechanism of action of this cytotoxin have appeared. Its presence should be confirmed, and, if it is found, its distribution among gonococcal isolates should be evaluated. Its mode of action should also be determined.

Studies that have investigated the structure and function of the outer membrane on protein (OMP) protein I (PI) (5, 6, 43, 45), however, provide a better example of how a gonococcal protein might modulate PMN function. PI is known to directionally insert into eucaryotic membranes, and this action may have dire consequences for PMNs (43, 64, 135). For instance, PI depolarizes the PMN membrane, resulting in inhibition of degranulation; this suggests that it could influence PMN structure and function. Since intracellular killing of gonococci requires the development of a phagolysosome (25), the inhibition of degranulation would prohibit the release of toxic compounds into the phagocytic vacuole. This may help to explain why some intraphagosomal gonococci resist the antimicrobial action of lysosomal components (see below).

Opsonization

What role do opsonins (12) play in phagocytosis of gonococci in vitro, and are opsonins produced and active in vivo? Gonococcal variants possessing certain PII proteins are readily phagocytized and killed in vitro (33, 53, 54, 83, 84, 129). With these strains, opsonins present in serum may have little effect on the outcome of gonococcus-PMN interactions. On the other hand, gonococci lacking PIIs or possessing nonadherent PIIs or, perhaps, strains displaying certain pilus chemotypes are not recognized by PMNs in the absence of opsonins.

Fresh human serum contains opsonic anti-PI immunoglobulin G (94, 95). Fresh convalescent-phase human serum samples collected during localized, disseminated, and uncomplicated gonococcal infections are opsonic and contain immunoglobulin G (11, 62) to most surface-exposed components, including PI (34, 63), PII (97), PIII (63, 86), the iron-regulated proteins (34), pili (34, 102), H.8 (4), and as yet uncharacterized components (34, 136). Such antibodies are also present in cervical and urethral secretions to various degrees (47, 65, 124).

In human PMN chemotaxis studies, Densen et al. (24) showed that complement deposition on disseminated isolates was less than and slower than that observed on local isolates, when incubated in 10% fresh human serum. Heat- or ethylenediaminetetraacetic acid (EDTA)-treated serum inhibited complement deposition on all strains and abrogated the subsequent release of the chemoattractant, C5a. Ross and Densen (92) further showed that serum-sensitive strains are more rapidly and completely ingested and killed by human PMNs than are serum-resistant strains in nonbactericidal C8-deficient serum or C8-depleted normal serum. More specifically, phagocytic killing of serum-resistant strains was unaffected by serum; in the presence of PMNs, it retained about 65% of its viability in the presence of 0.01 to 10% serum. In contrast, phagocytic killing of serum-sensitive strains was strictly dependent on the serum. An alternative explanation of the data, however, is that the representative serum-sensitive strain was consistently PII^-, whereas the serum-resistant strain was PII^+, thus confounding the interpretation of the results. Others find no differences in the phagocytic killing of unopsonized, nonpiliated PII^+ strains of serum-sensitive and serum-resistant strains (S. H. Fischer and R. F. Rest, unpublished data).

Opsonophagocytosis therefore appears likely in vivo, although we are not aware of studies investigating such processes. It is unknown which of the apparent opsonins is the most relevant for protection of the host.

SURFACE EVENTS

Gonococcal Surface Components Implicated in Gonococcus-PMN Interactions

Antiphagocytic activities and components. (i) PI. In a series of elegant studies, Blake and co-workers showed that PI translocated from the gonococcal OM to erythrocyte and artificial membranes (135) and associated with PMNs (64). PI forms ion channels in these membranes and selectively inhibits exocytosis from human neutrophils without inhibiting superoxide generation (43). Whether PI affects the ability of gonococci to survive neutrophil killing is not known.

(ii) Pili. Although pili are unquestionably important in initial interactions with host epithelial cells and in initiating gonococcal infection (46, 51, 52, 58, 111, 116, 118), their role in gonococcal interactions with PMNs remains open to question, and older data and conclusions must be reevaluated. With few exceptions, earlier studies showed that piliated gonococci (from type 1 or type 2 colonies, as originally described by Kellogg et al. [51, 52]) resisted neutrophil phagocytosis, whereas nonpiliated organisms, from colony type 3 or type 4, were ingested (27, 41, 58, 71, 80, 122, 123). However, more recent studies, and some earlier ones, have used gonococci with a better-defined PII content, as determined by a combination of colony morphology (59, 115), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane preparations (33, 54, 83, 112–116, 128, 129). The results of these studies have suggested that in the presence of PII, pili have little effect on the degree to which gonococci adhere to, stimulate, or are killed by human PMNs. Thus, PII appears more important than pili in mediating gonococcal interactions with PMNs, including adherence and modulation of phagocytosis (59, 60).

The story, however, is not complete. Pili undergo structural, antigenic, and functional alterations owing to antigenic variation (40, 103). There are no reports about the interaction of gonococcal pilus variants with PMNs. It is known that different pilus types confer upon gonococci different adherence properties for epithelial cells in culture (61). Recent information suggests that the interaction of gonococci may not be mediated by the major pilus subunit, pilin, but rather by as yet poorly defined pilus-associated proteins (67, 109) or proteins coexpressed in the complex transition from colony type 1 to colony type 4 (55). The molecular mechanisms by which pili mediate gonococcal interactions with human PMNs is far from completely understood, but it

is known (117) that piliation does not influence phagocytic killing.

(iii) Less well-defined events and components. In vitro growth of nonpiliated opaque (PII$^-$) gonococci in the presence of heat-inactivated normal human serum decreased their association with PMNs (9), reinforcing the observed need for opsonins to obtain efficient phagocytic killing. Gonococci isolated directly from human material or from guinea pig subcutaneous chamber fluid resisted phagocytic killing to a greater extent than did those grown in vitro (18, 126, 127, 134). In vivo resistance factors might be lost or altered upon subculture, as is the case for the antigenic and chemical structure of LPS (23, 96). Rosenthal et al. (91) identified an EDTA-sensitive antiphagocytic surface component of gonococci that has not been further studied. In addition to resisting phagocytosis or intracellular killing, gonococci might elaborate components toxic to neutrophils (19, 21, 43). Thus, from metabolic processes to surface structures and released toxic materials, gonococci possess a wide array of antineutrophil systems.

Adhesins and phagocytosis-promoting activities and components. (i) PII. There are at least three reasons why many early studies investigating the role of pili and other gonococcal surface components in the interaction with PMNs should be reevaluated. First, contrary to original observations and misconceptions, the possession of a particular PII does not necessarily confer upon gonococci the ability to form opaque colonies. Thus, transparent colonies can contain PII$^+$ gonococci, or, conversely, PII$^+$ gonococci can form transparent colonies (59, 115). Second, not all PII proteins confer upon gonococci the ability to adhere to, stimulate, and be phagocytically killed by PMNs (33, 60). Thus, colony opacity and adherence are unrelated, except under very well defined conditions. The leukocyte association protein (LAP) described by Swanson et al. was most probably a "transparent" PII, which is why its initial identification was so elusive (see below). Indeed, leukocyte association protein shares peptides with certain PIIs (114). Third, considering the high rate of pilin and PII antigenic variation (3, 40, 103) and the practical experience of many investigators, it is doubtful that the piliated and nonpiliated "isogenic" variants used in earlier studies possessed the same PII (or pilin subunits) throughout the period of study.

PMNs and gonococci are found in close association in vivo (31, 68, 72, 130, 131). They also adhere to each other in vitro in the absence of antibody. The leukocyte association protein, subsequently identified as a PII (114), was the first surface component other than pili to be studied in the adherence of gonococci to human PMNs. In the absence of opsonins, piliated or nonpiliated gonococci lacking PII adhered poorly to PMNs, were not engulfed or killed by PMNs, and induced only a low-level PMN oxidative response, compared with those possessing PII (33, 84, 129). Interestingly, PII also appeared to confer upon gonococci the ability to be phagocytically killed by *Trichomonas vaginalis* (32).

Which part of the PII molecule is involved in binding to PMNs? The various PII molecules synthesized by a particular strain generally have identical amino acid sequences except for a short semivariable (SV) region near the amino terminal and two longer hypervariable regions (HV1, [about 20 amino acids], and HV2, [about 35 amino acids]) in the carboxyl half of the PII molecule (20, 107). There are several variants of each HV region, e.g., HV1-1, HV1-2, HV1-n, and HV2-1, HV2-2, HV2-n. Differences in the SV region are limited to small changes, whereas the HV1 and HV2 regions show essentially no homology between or within themselves.

Several, but not all, monoclonal antibodies (whole or Fab fragments) directed against epitopes in HV2 block the ability of PII$^+$ gonococci to adhere to and stimulate PMNs (84, 129; C. Elkins and R. F. Rest, submitted for publication). The HV1 region does not seem to be directly involved in binding, since variants FA 1090 PIIa, which does not bind to PMNs, and FA 1090 PIIb, which does bind to PMNs, vary only in their HV2 region (20, 33; Elkins and Rest, submitted). Besides possessing different HV2 regions, PIIb (the adherent PII) possesses seven amino acids immediately adjacent to the HV2 region that PIIa (the nonadherent PII) lacks (20). Thus, an area near HV2 may be involved in gonococcal adherence to PMNs.

It has been suggested that PII mediates lectinlike interactions of gonococci with PMNs (84) and that opacity-associated PIIs bind gonococcal LPS (7). However, carbohydrate-specific binding of purified PII to HeLa cell membrane proteins was not observed (2). Such experiments have not been reported with proteins from PMN membranes. In addition, some of the observations by Rest et al. (84) concerning the ability of mannose to inhibit gonococcus-induced PMN chemiluminescence may have been artifactual, since further studies revealed that mannose inhibits PMN oxidative metabolism in response to several stimulating agents (82). PIIs have been strongly implicated in adherence to epithelial cells (1, 48, 110). Whether different PIIs modulate adherence to different cell types remains to be determined.

(ii) LPS. In adherence studies with PMNs and piliated gonococci (whose PII content was not defined), Kinane et al. (53) showed that additional lectinlike interactions might occur, with the lectin on the neutrophil and the carbohydrate on the gonococcus. This suggests a role for LPS, or an unidentified gonococcal glycolipid or glycoprotein, in the adherence of gonococci to PMNs. With the recent description of frequent antigenic, structural, and functional changes (similar in frequency to pilin and PII antigenic variation) in the gonococcal LPS, such observations take on a new significance (26, 96).

Periodate oxidation abrogates gonococcal adherence to PMNs (54; Elkins and Rest, submitted). Since this is true for nonpiliated, PII$^+$ organisms, it can be concluded that PII, which does not contain carbohydrate, contains a periodate-sensitive portion, perhaps containing the periodate-sensitive amino acid serine, threonine, or tyrosine (39, 132). Alteration of LPS by periodate does not appear to be responsible for these observations, since a gonococcal LPS mutant, WS 1 (100), which contains few or no hexoses, also binds to PMNs in a strictly PII-dependent manner (Elkins and Rest, submitted). Further studies have shown that binding of nonpiliated PII$^+$ gonococci to PMNs (i) is temperature dependent, with no binding occurring at 0 to 5°C; (ii) is independent of gonococcal viability, since gonococci killed by gentamicin or ultraviolet light bind as well as viable gonococci (Elkins and Rest, submitted); and (iii) is inhibited by treatment of gonococci with trypsin, chymotrypsin, proteinase K, or V8 protease.

Characterization of Gonococcus-PMN Interactions and Possible Receptors

Few studies have characterized the molecular mechanisms involved in gonococcus-PMN interactions or investigated the structure of the PMN receptor(s) for gonococcal

adhesins. Adherence of gonococci to PMNs is not inhibited by protease treatment of PMNs (Elkins and Rest, submitted). On the contrary, PMNs treated with proteinase K show a 5- to 10-fold increase in the binding of gonococci (C. Elkins, C. Farrell, and R. F. Rest, unpublished data). Several PMN secretagogues, including phorbol myristate acetate, the calcium ionophore A23187, and the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (in the presence of 5 g of cytochalasin per ml), all increase, in a dose-responsive manner, the ability of PMNs to bind nonpiliated PII^-, but not PII^+, gonococci. Furthermore, cells of the promyelocytic cell line HL-60 do not bind such gonococci, regardless of their state of differentiation or the presence of secretagogues (Elkins et al., unpublished).

Recently, Stromberg et al. (109) showed that gonococci, regardless of piliation or PII content, adhere to several glycolipids, including lactosyl-, isoglobotriaosyl-, gangliotriaosyl-, and gangliotetraosylceramides. Whether these glycolipids play a role in binding of gonococci to PMNs or to epithelial cells is not known, although the last two, also known as asialo-GM_2 and asialo-GM_1, respectively, have not been identified in neutrophils (36, 37, 121). Such results gain importance in light of new studies defining gonococcal pilus-associated (nonpilin) proteins (68). Perrollet and Guinet (78) described a 65-kilodalton gonococcal outer membrane protein with maltose- and D-glucosamine-binding activities. Its identity with OMP-macromolecular complex (44) and its role in adherence to host cells is unknown. Wiseman et al. (133) have suggested that piliated (colony type 1) gonococci agglutinate human erythrocytes (13, 57, 80) as a result of interactions with D-galactose-containing erythrocyte surface-exposed glycoconjugates. In studies with lectin-resistant Chinese hamster ovary cells (29), Gubish et al. (42) observed that isolated pili adhere to parental cells much better than to the mutant cells lacking sialic acid, galactose, and *N*-acetylglucosamine in their cell surface glycoconjugates. Results obtained by Pearce and Buchanan support the idea that gonococcal pili adhere to glycosphingolipids of host cells (77). Such interactions are not inhibited by mannose or other monosaccharides. A common thread to all these studies is the dependence of gonococcal binding to host cells on galactose-containing glycoconjugates, probably glycolipids.

INTRACELLULAR EVENTS

Gonococcal Survival within PMNs

Whether gonococci survive within PMNs has been the subject of controversy, with no clear-cut answer yet available. Perhaps no other issue in this field has generated more conflicting data. The intracellular fate of gonococci is clearly an important issue, given that protective antibodies resulting from vaccination will probably have to be opsonic.

Densen and Mandell (25) established that phagolysosomal formation was required for phagocytic killing of gonococci. No evidence to date has been published that intracellular gonococci inhibit phagosome-lysosome fusion or that certain gonococci escape from the phagolysosome. Accordingly, if gonococci are to survive within PMNs, they apparently must resist antimicrobial systems that operate in the phagolysosome.

What is the conflicting evidence regarding the intracellular fate of gonococci, and how can the controversy be explained? First, it is important to note that differences in phagocytic killing assays (monolayer versus suspensions of PMNs), strain heterogeneity (isolates from localized versus disseminated gonococcal infection), the efficiency with which attached gonococci are eliminated by antimicrobial agents, and gonococcal growth conditions (log-phase versus stationary-phase gonococci, broth grown versus agar grown, aerobic versus anaerobic conditions) are likely to influence the rate and extent of killing. Hence, assessment and comparison of survival data from different laboratories are difficult. Second, it is very important to remember that phagocytosis assays performed in the laboratory may only slightly resemble the events occurring in vivo and the environmental conditions in tissues or blood. Although this is always a problem in research on microbial pathogenesis, it is especially true for gonococci, given the highly variable surface structures and response to environmental stresses.

The greatest proponents of the intracellular survival of gonococci are Smith and co-workers in England. In a series of papers (17–19, 73–76, 126, 127, 134) starting in the mid 1970s and continuing to the present, this group presented data suggesting that a percentage of gonococci not only survive but also replicate within PMNs; replication was inferred on the basis of the capacity of penicillin to kill ingested gonococci (126), with the rationale that penicillin kills only growing bacteria. Moreover, such intracellular survival of gonococci is enhanced if gonococci are first cultivated in vivo by using the guinea pig chamber model (127). Such gonococci are postulated to more closely resemble, in an antigenic and physiological sense, gonococci growing in humans. In support of this, Parsons et al. (73–75) recently reported that a surface-exposed 20-kilodalton OMP promotes intracellular growth and survival of gonococci. This OMP contains fatty acids (ca. 5.7% of the total protein content) and is rich in glutamic acid. Evidence that this lipoprotein confers on gonococci increased resistance to intracellular killing is based, in part, on the observation that a monospecific antiserum against it reduces gonococcal resistance to intracellular killing. Clearly, this antigen is of interest and should be studied in more detail at both the biochemical and molecular levels. How it confers gonococcal resistance to killing systems in phagolysosomes will be of interest. Of additional interest is how in vivo growth stimulates its synthesis.

Not all researchers have observed intracellular survival and growth of gonococci (25, 27, 41, 58, 83, 95, 122). These investigators observed that when an inoculum of gonococci was allowed to interact with PMNs in either monolayers or suspension, the ingested gonococci were rapidly killed. Any gonococci that survived, however, were suspected to be attached to PMNs in a manner that enabled them to resist the extracellular antimicrobial agents typically added to eliminate them. Accordingly, the question of intracellular survival of gonococci remains controversial. However, in our opinion, the major difference between the studies by Smith and co-workers and those of others is that the former group has often used gonococci directly from clinical material, while the latter has not.

The observations of Smith and co-workers may ultimately prove to be correct in that survival of gonococci in vivo is greater than that observed in in vitro phagocytosis assays. Recent support for the concept that some intracellular gonococci survive comes from the work of Casey et al. (16). In a study to test whether anaerobically maintained PMNs killed gonococci as effectively as aerobically maintained PMNs did, they used pyocin 103 to kill piliated gonococci attached to PMNs. This approach was needed to rigorously distinguish attached from ingested gonococci (120). Pyocin 103 exhibits potent gonococcidal action and is not phagocytized,

thus proving to be an effective extracellular killing agent. They observed that approximately 2% of the total ^{14}C-labeled, piliated gonococci ingested by PMNs survived for at least 165 min. Two important conclusions can be drawn from these experiments. First, in the absence of oxygen, PMNs kill 98% of the ingested gonococci, thereby implicating oxygen-independent antimicrobial mechanisms as contributing significantly to intraleukocytic killing of gonococci. Second, despite such effective intraleukocytic killing of gonococci, a small but reproducible number survive.

Oxygen-Independent Killing of Gonococci by PMNs

The finding (16) that an oxygen-independent antimicrobial mechanism(s) contributed significantly to intraleukocytic killing of gonococci was consistent with the earlier work of Rest et al. (83), who showed that PMNs obtained from normal healthy donors and patients with chronic granulomatous disease displayed identical phagocytic killing capacities. This observation was significant because chronic granulomatous disease renders phagocytes incapable of generating toxic O_2 radicals necessary for oxidative killing systems (reviewed in part in reference 104). Hence, intraleukocytic killing of gonococci by chronic granulomatous disease PMNs is due entirely to a nonoxidative process.

It is also important to note that neutrophils derive all their energy through glycolysis (104). Hence, an anaerobic environment places no constraints on their energy needs. In fact, it is tempting to speculate that the lack of toxic oxygen radicals within a PMN may prolong its life, or at least make it more enjoyable.

The illuminating work of Britigan et al. (9–11) suggests that even under aerobic conditions, gonococci may be killed by normal PMNs by nonoxidative processes. They showed that gonococci exposed to serum- or phagocyte-derived lactate rapidly consumed the available molecular oxygen. Thus, gonococcal oxygen consumption would deplete the oxygen available to PMNs in a microenvironment, thereby reducing their capacity to mount an oxidative burst.

The emerging realization that gonococci survive and grow under anaerobic or hypoxic conditions (15, 49, 50, 56) and coexist with strict anaerobes in vivo (35) is another reason why the nonoxidative antimicrobial system of PMNs is an important topic for study. Largely through the work of Clark and co-workers (55, 56), we know that anaerobic conditions can be tolerated if gonococci are provided with a surrogate electron acceptor such as sodium nitrite and that anaerobiosis regulates the synthesis of gonococcal OMPs. Hence, gonococci growing anaerobically in vivo may little resemble the same strain propagated in vitro in broth or on agar in an oxygenated environment. How might anaerobic conditions influence gonococcal pathogenesis, given that the repertoire of surface antigens might change? First, in the absence of a surrogate electron acceptor, gonococci are in a state of bacteriostasis. Casey et al. (15) found that this resulted in gonococcal resistance in vitro to antimicrobial granule proteins. Addition of sodium nitrite, however, permitted growth in anaerobic broth and resulted in a level of granule protein susceptibility similar to that in aerobically grown gonococci. This is consistent with the earlier work of Rest and coworkers (14, 81, 88, 89), who showed that granule proteins kill actively growing gonococci better than they kill stationary-phase gonococci.

The antimicrobial action of certain cationic granule proteins is thought to be a major determinant of nonoxidative killing of bacteria within phagolysosomes (104). These toxic proteins are stored in the cytoplasmic granules and are released into the vacuole, where they rapidly bind to the microbial surface. To date, several proteins have been isolated from extracts of granules and shown to possess antimicrobial action in vitro (38, 69, 98–101, 104). Those possessing antigonococcal activity are CAP 37, CAP 57, and cathepsin G (98–101). The low-molecular-weight defensins (38), having potent antibioticlike activity against several bacteria, fungi, and viruses, have surprisingly little (if any) antigonococcal activity in vitro.

Shafer et al. (98–101) have studied extensively the antigonococcal activity of cathepsin G and concluded that it is a likely participant in the killing of gonococci in phagolysosomes. Although this protein is a serine protease with chymotrypsinlike specificity (69, 93), it kills gonococci and other pathogens by a mechanism independent of proteolysis; neither boiling for 5 min nor treatment with the potent serine protease inhibitor diisopropyl fluorophosphate diminishes the bactericidal action of cathepsin G (69, 70, 100, 101). Thus, one can exclude proteolytic damage to the outer membrane as a mechanism of killing. The highly cationic nature of cathepsin G (pI > 12.5) is a probable reason why it binds avidly and in a saturable manner to gonococci (98). A single diplococcus has approximately 10^5 binding sites for cathepsin G. These binding sites are masked by LPS, since a mutation which truncates LPS enhances gonococcal susceptibility to cathepsin G and increases the specific binding of cathepsin G 10-fold. Analysis of the saturable binding of cathepsin G to gonococci revealed a binding constant of 10^8 M^{-1}.

PI and PIII appear to bind cathepsin G (98, 99). This was first appreciated when the degradation of PI and PIII by enzymatically active cathepsin G was discovered (99). Such degradation was more extensive in an LPS mutant (WS 1) that exhibited hypersusceptibility to the lethal action of cathepsin G. More recent work with enzymatically inactive cathepsin G showed that antibody to cathepsin G coimmunoprecipitated cathepsin G, PI (serovar 1A1), PIII, and a 45-kilodalton OMP. This interaction between cathepsin G and gonococcal OMPs is also enhanced in the LPS mutant. However, recent studies (W. M. Shafer and V. C. Onunka, unpublished observations) show that loss of PIII by insertional mutagenesis (8) does not influence the level of gonococcal susceptibility to cathepsin G.

Although binding of cathepsin G or other antimicrobial proteins to the microbial surface is a necessary step in the antimicrobial process, it is probably not sufficient for killing. A postbinding event(s) leading to the death of the target microorganism is required. The nature of the event remains to be determined. Nevertheless, some progress has been made in determining the mechanism by which gonococci succumb to antimicrobial proteins. Gonococci incubated in the presence of lethal amounts of unfractionated granule extracts continue protein and nucleic acid synthesis (14) and retain respiratory activity, but are impaired in PG synthesis (88) and show a limited capacity to divide (89). This was evidenced by a reduced incorporation of radiolabeled glucosamine into PG during incubation of gonococci with granule extracts. Such gonococci exhibit altered septa, and their OMs have an amorphous appearance. Further evidence for an involvement of PG in the lethal mechanism of antibacterial proteins (including cathepsin G) is that a mutation (*penA2*) (22, 28, 105) known to modify (28) penicillin-binding protein 2 (PBP-2) (a transglycosylase-transpeptidase) involved in the terminal stages of PG synthesis (105) renders gonococci hypersusceptible to both crude granule extracts

(22) and cathepsin G (98–101). Hence, cathepsin G may exert its antigonococcal activity against gonococci by binding to the surface, thereby causing damage to the OM, which then permits interference with enzymes (such as PBP-2) involved in the terminal stages of PG synthesis. How does a mutation that decreases the binding of penicillin to PBP-2 increase susceptibility to cathepsin G? It appears that granule proteins inhibit the binding of radiolabeled penicillin to PBPs, suggesting that granule proteins such as cathepsin G may have significant affinity for PBPs (R. F. Rest, unpublished data). Thus, mutations that alter PBPs may enhance the binding of cathepsin G, thereby leading to an inhibition of PG synthesis.

Recently, an 870-base-pair complementary deoxyribonucleic acid sequence having the coding capacity for a full-length molecule of cathepsin G has been isolated (93), cloned into λ gt11, and subcloned behind a T7 ribonucleic acid polymerase promoter in pT7-6 (W. M. Shafer, G. Salvesen, and J. Travis, submitted for publication). With this plasmid expression system, recombinant cathepsin G has been produced in *Escherichia coli*. The isolated recombinant polypeptide has bactericidal activity against gonococci that is identical to that of the lysosomal cathepsin G. The fact that recombinant cathepsin G kills gonococci (and other cathepsin G-susceptible bacteria) indicates that the antibacterial domain(s) of cathepsin G resides within its primary structure. It should now be possible, through recombinant deoxyribonucleic acid and mutagenic techniques, to identify such "antimicrobial" domains of cathepsin G. This will be of great benefit in determining the mechanism of killing.

Degradation of Gonococci by Lysosomal Enzymes

Degradation of gonococcal antigens is of importance since surface antigens or potentially toxic substances (LPS and PG) may be altered. Such action could modify antigenicity or biologic activity. Only a few studies to date, however, have examined this topic.

Proteases (notably the neutral serine proteases elastase and cathepsin G) degrade a number of gonococcal OMPs. PIB and PIIs are susceptible to PMN elastase (30, 85). This occurs in vitro when highly purified elastase and gonococci or OM preparations are used and in situ when gonococci are present in phagolysosomes. Elastase also has the capacity to cleave highly purified pili with at least one cleavage site in or near the highly conserved CNBR-2 domain of pilin (106). Such cleavage of pili in antigen-presenting macrophages may alter the domains of pilin thought to be important in biologic and immunologic activity. Cathepsin G is a much weaker protease than elastase, but it can degrade PIII and the major iron-regulated OMP termed MIRP 37 (99). The PIA molecule appears to be somewhat resistant to cathepsin G, but the PIB subclass can be degraded (Shafer et al., unpublished). Other hydrolyses in granules may also participate in digestion of the gonococcal cell envelope. Certainly lysozyme (present in both specific and azurophil granules [104]) may have a role in degrading gonococcal PG in phagolysosomes. An unrelated lysosomal enzyme has been described by Striker et al. (108) that is activated by acidic conditions and appears to remove terminal glucosamine residues in PG. Since this enzyme is activated by acidic conditions, it probably performs a PG-modifying activity in the terminal stages of phagolysosome development when the environment is acidic. The number of lysosomal hydrolyses that can attack substrates like PG (108) may give rise to diverse types of PG fragments that are thought to be liberated in vivo and may regulate human physiology. In this respect, recent studies by Powers and Shafer (unpublished observations) indicate that cathepsin G can cleave PG fragments of strain RD5 liberated after digestion with lysozyme. Cathepsin G cleavage of PG was predicted, since molecular modeling of the active site of cathepsin G revealed that diaminopimelic acid fits into the active site.

What is the fate of the gonococcal antigens liberated from digested gonococci or modified after the antigens themselves have been degraded? Is their presence in tissues and/or fluids significant for the pathology of the different forms of gonorrhea? Do they have immunomodulatory activities? For instance, both gonococcal PG and LPS have toxic activities when prepared from bacteria grown under laboratory conditions. What are their activities in vivo after being processed by phagocytic cells? Answers to these questions are not available. However, this should be a fertile area of research in years to come, particularly since more information is becoming available regarding the structure of gonococcal LPS and PG, as well as the PMN hydrolyses that modify their structure.

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Genetic Loci and Linkage Associations in *Neisseria gonorrhoeae* and *Neisseria meningitidis*

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The purpose of this review is to present the currently available information on genetic loci that have been identified in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. We will include genes that were identified by mutations that alter the phenotype of the organism, if the gene was transferred into another genetic background, and genes whose products were well characterized, even if the genes were not identified by mutation. We also will include genes that were cloned and identified either by complementation of *Escherichia coli* mutations or by identification of the gene product.

GENETIC NOMENCLATURE

We will attempt to establish genetic nomenclatures for these genes, following the guidelines of Demerec et al. (29), i.e., assigning a three-letter lowercase designation for the gene family, followed by a capital letter, as appropriate. Genes that have been identified by complementation of well-characterized *E. coli* mutants will be given the corresponding genetic symbol, if appropriate. This is not meant to imply that the neisserial gene encodes the same enzyme as the *E. coli* gene, but, rather, that it encodes an enzyme that performs the same function in converting the substrate into the product. For cloned neisserial genes that were identified by the gene products, which are not found in *E. coli*, we will use the previously published nomenclatures, except for genes whose names have been changed by agreement of researchers attending the Sixth International Pathogenic Neisseria Meeting, October 1988. Genes that were identified and named in *N. gonorrhoeae* should be given the same gene symbol if they are subsequently found to occur in *N. meningitidis*, and vice versa.

Since *N. gonorrhoeae* and *N. meningitidis* contain several copies of closely related genes, a special nomenclature has been agreed upon to identify these genes. This consists of giving the genetic symbol following by a subscript notation that identifies the strain containing the gene with a designation of the allele number. For example, the protein IIb gene from *N. gonorrhoeae* FA1090 would be $opa_{FA1090-2}$. Genetic designations for these multicopy genes, e.g., as *opaA*, are not to be given until the gonococcal chromosome has been genetically or physically mapped and the location of the gene on the map has been determined.

New genetic symbols or proposed revisions of old nomenclature are to be approved by Virginia L. Clark, Department of Microbiology and Immunology, Box 672, School of Medicine and Dentistry, University of Rochester, Rochester, New York. She will also assign allele numbers to designate mutations in specific genes upon written request. It is hoped that this coordination in genetic nomenclature for *N. gonorrhoeae* and *N. meningitidis* will prevent confusion that may arise if different laboratories assign their own genetic designations.

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N. GONORRHOEAE LABORATORY STRAINS

Comparisons of results from different laboratories are sometimes difficult owing to the use of different strains that may vary in their genetic composition. Therefore, we would like to briefly review the laboratory strains that have been used in these studies. The most commonly used laboratory strains are listed in Table 1, along with available information regarding their history and genetic markers.

GENETIC MARKERS IN *N. GONORRHOEAE*

Gonococcal genetic markers identified by transformation, by characterization of the gene product, or by cloning are listed in Table 2, and the limited genetic map is shown in Fig. 1. It should be noted that the genetic map contains a number of ribosomal protein mutations (*str*, *fus*, *tet*, *cap*, and *spc*) and a subunit of ribonucleic acid polymerase (*rif*); ribosomal protein genes have been shown to be clustered in other organisms (6), so it is not surprising to find them clustered in *N. gonorrhoeae*. The other genes that have been shown to be linked in this region are probably involved in peptidoglycan biosynthesis (*ampD*, *ampC*, *ampB*, *ampA*, and *penB*) or outer membrane structures (*por*, *sac-1*, *sac-3*, *mom*, and *tem*). The genes linked to this region that encode enzymes involved in monomer synthesis (*ura*, *argE*, and *hyx*) are found flanking the region, rather than within it.

In addition to the limited genetic map, which represents approximately 3% of the gonococcal chromosome (19), physical maps linking various gonococcal genes have been generated. The largest portion of the gonococcal chromosome that has been physically mapped, using chromosome walking techniques, is 50 kilobase pairs and contains the two pilus expression sites, a pilus silent gene region, and a protein II gene (75, 113).

Little is known regarding genetic organization and control of gene expression in *N. gonorrhoeae*. No repressors or activators have been identified by either classical genetic or gene cloning techniques. However, repression of outer membrane protein synthesis by iron (123) and oxygen (25) suggests that regulatory proteins do exist in *N. gonorrhoeae*. Additionally, two genes, *pilA* and *pilB*, that act in *trans* to regulate the pilin promoter have recently been described (117). The *pilB* gene product represses pilin expression, whereas the *pilA* gene product activates the pilin promoter.

No evidence exists that gonococcal genes are organized into operons, with multiple genes under the control of a single promoter. The cloned *proA* and *proB* loci are contiguous but are under the control of separate promoters (110). It is possible that most gonococcal genes contain their own promoter, analogous to the genetic organization of *Pseudomonas aeruginosa* (54) rather than *E. coli* (6).

TABLE 1. Characteristics of common laboratory strains of *N. gonorrhoeae*

Strain	Genotype and/or relevant phenotypic characteristics[a]	Comments[a]	Reference(s)[b]
ATCC 19424	Met⁻ Dam⁺, PIA-1,2	Type strain	119, 121
F62[c]	*proA sac-3* (Sac⁵), Lf⁺ Dam⁺, PIB-7, antibiotic susceptible	More readily transformed than other gonococcal strains	38, 58, 78, 97, 110, 119, D
FA19[d]	Prototrophic, *sac-1 sac-3* (Sac⁵), Lf⁺, PIA-1, methylation of the DNA sequence GGCC, antibiotic susceptible	Used for much of the genetic mapping of the gonococcal chromosome	78, 93, 97, 105, C, E
FA1090[e]	Pro⁻ Str[r] but susceptible to other antibiotics, Sac⁵, PIB	Used for studies of protein II antigenic and phase variation; monoclonal antibodies specific for various protein II types have been made	9, B, C
$MS11_{ms}$[f]	Prototrophic; *pilE1*⁺ *pilE2*⁺, PIB-9, Str⁵; methylation of DNA sequences PuGCGCPy, CCGCGG, and GCCGGC; restriction endonuclease activity against DNA sequence GCCGGC	Used for studies of pilin expression and antigenic variation	28, 75, A, C, E
P9	Dam⁺; methylation of DNA sequences PuGCGCPy, GGCC, CCGCGG, GCCGGC, GGNNCC; restriction endonuclease activity against DNA sequences GGCC, CCGCGG	Used for studies of pilin antigenic variation; isogenic variants of P9 have been isolated that produce immunologically distinct pili	28, 65, 84, 114
1342[g]	Arg⁻, PIB-1	Prototype strain for LPS serotype GC_1	1–4, 38
1291[g]	Prototrophic, PIB-1	Prototype strain for LPS serotype GC_2	1–4, 38
4505[g]	Prototrophic, PIB-3	Prototype strain for LPS serotype GC_3	1–4, 38
8551[h]	Pro⁻, PIB-4	Prototype strain for LPS serotype GC_4	1–4, 38, 66
PID2	Prototrophic, PIB-3	Prototype strain for LPS serotype GC_5	3, 4, 38
3893	Prototrophic, PIA-1	Prototype strain for LPS serotype GC_6	3, 4, 38
JW31R	Prototrophic, pyocin resistant, serum sensitive	Pyocin-resistant mutant of strain JW31 which has lost the LPS variable and serotype antigens but has retained the common LPS determinants	81

[a] Symbols: *proA*, requirement for proline; *sac*, resistance to the bactericidal activity of normal human serum; Lf, utilization of lactoferrin as a sole source of iron; Dam, methylation of adenine in GATC sequences; PI, protein I serovar (118); Str[r], streptomycin resistance; *pilE*, pilin expression locus; Arg⁻, Met⁻, and Pro⁻, requirement for arginine, methionine, or proline, respectively; LPS, lipopolysaccharide.

[b] Numbers refer to Literature Cited. Letters refer to abstracts or personal communications from the listed individual(s): (A) R. H. Chien, D. C. Stein, H. S. Seifert, K. Floyd, and M. So, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, K41, p. 213; (B) J. G. Cannon; (C) N. Carbonetti; (D) P. F. Sparling; (E) D. C. Stein.

[c] *N. gonorrhoeae* F62 was isolated from the urethra of a female at the Fulton County Health Department, Atlanta, Ga., in 1962. Human volunteers were infected with this strain to demonstrate that virulence was genetically linked to clonal variation (58).

[d] *N. gonorrhoeae* FA19 is a urogenital isolate from the Far East (P. F. Sparling, personal communication). It is strain Ceylon 3 from the collection of A. Reyn and was obtained from her by P. F. Sparling in 1970. It was lyophilized by A. Reyn in 1962. The antibiotic susceptibilities of FA19 are typical of those from strains isolated in the preantibiotic era and are as follows (expressed in micrograms per milliliter): penicillin, 0.007; tetracycline, 0.25; chloramphenicol, 0.5; erythromycin, 0.25; rifampin, 0.12; fusidic acid, 0.12; Triton X-100, 0.5; acridine orange, 100; and crystal violet, 4.0 (93, 94, 105).

[e] *N. gonorrhoeae* FA1090 is a cervical isolate from a patient with disseminated gonococcal infection and was isolated in North Carolina in 1976 (J. G. Cannon, personal communication).

[f] *N. gonorrhoeae* MS11 is from the collection of E. Gotschlich, The Rockefeller University, New York, N.Y., and was initially isolated at the Mount Sinai School of Medicine, New York, N.Y., in 1970. $MS11_{ms}$ was obtained from the collection of E. Gotschlich by M. So and T. F. Meyer (77). *N. gonorrhoeae* $MS11_{mk}$ differs from $MS11_{ms}$ in that the *pilE2* locus is deleted. $MS11_{mk}$ was obtained by J. M. Koomey and J. Swanson from stocks supplied by G. Schoolnik, who serially passaged them from the collection of E. Gotschlich (116).

[g] Isolated in Buffalo, N.Y. (38).

[h] Urogenital isolate from Norway (66).

CLONED *N. GONORRHOEAE* GENES AND CODON USAGE

The ability to clone genes that are readily expressed in *E. coli* has greatly enhanced the identification of genetic loci in *N. gonorrhoeae*. The genes that have been cloned from *N. gonorrhoeae* are listed in Table 3. A variety of vectors, including lambda bacteriophage derivatives, plasmids, cosmids, and phasmids, have been used to clone gonococcal genes. The plasmid pBR322 has been used effectively for cloning genes that are not lethal to *E. coli*, whereas lambda gt11 has proven effective for cloning portions of the protein I (*por*) gene and for cloning the protein III (*rmp*) gene. It appears to be necessary to use restriction-deficient *E. coli* strains ($McrB^-$ $HsdR^-$), in most cases, to obtain high cloning efficiencies (13, 92, 109, 114).

Several of the cloned gonococcal genes have been sequenced, and the *N. gonorrhoeae* codon usage for these genes is listed in Table 4. Codon usage tables are useful for identifying protein-coding regions in a nucleotide sequence (47, 108), locating deoxyribonucleic acid (DNA) sequencing errors (47, 108), and constructing oligonucleotide probes from amino acid sequences. In *E. coli* there is a different pattern of codon usage depending on the level of expression of a particular gene in a cell (46, 48). Even though the 11 gonococcal genes which were examined could all be considered highly expressed genes (i.e., present in more than 10 protein molecules per cell, as opposed to the small number of regulatory protein molecules per cell), the *N. gonorrhoeae* codon usage pattern most resembles that of *E. coli* non-highly-expressed genes, such as the lambda and *lac* repressors. This difference in codon usage cannot be explained by differences in guanine-plus-cytosine content, since the guanine-plus-cytosine content of *N. gonorrhoeae* is similar to that of *E. coli*. The pattern of codon usage in the pilin genes is most different from that in the other gonococcal genes examined, with a much more biased use of cytosine in codon position 3. Codons which are rarely used by *N. gonorrhoeae* are glycine (GGG), arginine (AGG, AGA, and CGA), and leucine (CTA).

TABLE 2. Genetic markers of *N. gonorrhoeae*

Gene symbol	Mnemonic	Linkage association[a]	Alternative gene symbols; phenotypic trait affected	Reference(s)[b]
aniA	Anaerobically induced		Major anaerobically induced outer membrane protein (Pan 1)	25
ampA	Ampicillin	*cap*	Low-level resistance to ampicillin	55
ampB	Ampicillin	*str, fus*	Low-level resistance to ampicillin	55
ampC	Ampicillin	*rif, str*	Low-level resistance to ampicillin	55
ampD	Ampicillin	*rif*	Low-level resistance to ampicillin	55
argA	Arginine		Complementation of *argA* mutation of *E. coli* NK5992 and W3421; acetylglutamate synthetase	23, I
argB	Arginine		Complementation of *argB* mutation of *E. coli* 30SOMA4	I
argE	Arginine	*penB, hyx*	*argJ*; complementation of *argE* mutation of *E. coli* AB1157 and AT2538; ornithine acetyltransferase	5, 22, 23, 98, F, I
argF	Arginine		*argI*; complementation of *argI* mutation of *E. coli* N166 and *argF argI* mutation of *E. coli* N134; ornithine transcarbamylase	22, 98, F, I
argG	Arginine		Complementation of *argG* mutation of *E. coli* UQ27	23, I
cap	Chloramphenicol	*tet, spc*	*cam, chl*; low-level resistance to chloramphenicol	68, 93, 105
carA		*carB*	*car-1*; requirement for ornithine or arginine; complementation of *carA* mutation of *E. coli* FP178; small subunit of carbamoyl-phosphate synthetase	22, 99, I
carB		*carA*	*car-2*; requirement for ornithine or arginine; complementation of *carB* mutation of *E. coli* JEF8; large subunit of carbamoyl-phosphate synthetase	22, 99, I
dcmA[c]	DNA cytosine methylase		DNA methylase M.*Ngo*PI; recognition sequence 5′-PuGCGCPy-3′	28, 114
dcmB[c]	DNA cytosine methylase		DNA methylase M.*Ngo*PII; recognition sequence 5′-GGCC-3′	28, 114, 115
dcmD[c]	DNA cytosine methylase	*dcrD*	DNA methylase M.*Ngo*MI; recognition sequence 5′-GCCGGC-3′	28, D, E
dcmE[c]	DNA cytosine methylase	*dcrE*	DNA methylase M.*Ngo*BIII; recognition sequence 5′-GGNNCC-3′	28, J
dcrD[c]		*dcmD*	Restriction endonuclease R.*Ngo*MI; recognition sequence 5′-GCCGGC-3′	28, D, E
dcrE[c]		*dcmE*	Restriction endonuclease R.*Ngo*BIII; recognition sequence 5′-GGNNCC-3′	28
dud	DNA uptake deficient		Failure to take up DNA into a deoxyribonuclease (DNase)-resistant state; abnormal colony morphology	10a, C
env-1	Envelope	*env-3*	*envA*; nonspecific increased sensitivity to antibiotics, dyes, and detergents; complete phenotypic suppression of *mtr* and *penB*; 40% decrease in cross-linking of peptidoglycan; fivefold reduction in quantity of 52,000 mol wt outer membrane protein	35, 50, 71, 94, 106
env-3	Envelope	*env-1*	*envB*; nonspecific increased susceptibility to antibiotics, dyes, and detergents; 20% decrease in cross-linking of peptidoglycan	35, 50, 71, 94, 106
env-10	Envelope		Increased susceptibility to drugs, dyes, and detergents	96
ery	Erythromycin	*spc*	Low-level resistance to erythromycin	68
fbp	Iron-binding protein		Structural gene for an iron-binding outer membrane protein (37,000 daltons)	80, A
frp	Iron-repressible protein		Family of outer membrane proteins expressed under conditions of iron starvation	85, 123
fhuB	Ferric hydroxamate uptake		Complementation of *fhuB* mutation of *E. coli* BN3307 and BU736	124
fud	Iron uptake deficient		Deficient in iron acquisition	*
fus	Fusidic acid	*str, tet*	Resistance to fusidic acid	104
hsp	Heat shock protein		Family of proteins whose expression increases after a shift up in temperature	59
hyx	Hypoxanthine	*argE*	Requirement for hypoxanthine	5, 72
iga	Immunoglobulin A		Immunoglobulin A protease	61, 90
laz	Lipid-modified azurin		Azurin-related outer membrane protein; recognized by monoclonal antibody H.8	42, 43

Continued on following page

TABLE 2—*Continued*

Gene symbol	Mnemonic	Linkage association[a]	Alternative gene symbols; phenotypic trait affected	Reference(s)[b]
ldhA	Lactate dehydrogenase		Membrane-bound lactate dehydrogenase	B
ldhB	Lactate dehydrogenase		Soluble lactate dehydrogenase	B
lip	Lipoprotein		Outer membrane protein recognized by monoclonal antibody H.8	12, 53, L
lps-1	Lipopolysaccharide synthesis		*los*; produces new lipopolysaccharide band in *E. coli* HB101 which is recognized by antigonococcal antiserum	86
lps-2	Lipopolysaccharide synthesis		*los*; biosynthesis of lipopolysaccharide; complementation of pyocin-resistant phenotype of *N. gonorrhoeae* FA5100	G
lps-3	Lipopolysaccharide synthesis		*los*; biosynthesis of lipopolysaccharide; complementation of pyocin-resistant phenotype of *N. gonorrhoeae* FA5100	G
mom	Modifier of *mtr*	*penB*	Phenotypic suppression of *mtr*; reduction of resistance to benzylpenicillin, oxacillin, erythromycin, and novobiocin mediated by *mtr*	100
mtr	Multiple transformable resistance		*ery-2*; low-level resistance to penicillin, tetracycline, erythromycin, chloramphenicol, rifampin, and fusidic acid and increased resistance to dyes and detergents; fivefold-increased amount of a 52,000-dalton outer membrane protein; 30% increase in cross-linking of peptidoglycan	40, 49, 50, 67–69, 103, 105, 106, 122
metB	Methionine		Complementation of *metB* mutation of *E. coli* χ342	89
nsr[d]	Nonspecific resistance		*mtr*; low-level resistance to antibiotics; does not alter the amounts of a 52,000-dalton membrane protein as does *mtr*	14, 19
ntr-2	Nontransformable		Inability to be transformed by plasmid or chromosomal DNA; normal DNase-resistant DNA uptake; normal colony morphology	10a, C
ntr-5	Nontransformable		Inability to be transformed by chromosomal DNA; normal DNase-resistant DNA uptake	10a, C
omc	Outer membrane complex		Structural gene for the outer membrane protein-macromolecular complex (76,000 daltons)	82, K
opa	Opacity	*pilE1*[e]	Outer membrane protein II structural gene	26, 74, 95, 113
oxiA	Oxygen induced		Oxygen-induced outer membrane protein (28,000 daltons)	25, 36, 37
pem	Penicillin modifier		Modifier gene affecting resistance to penicillin	121
penA	Penicillin		Penicillin-binding protein PBP-2; penicillin-susceptible enzyme involved in peptidoglycan synthesis at cell division; four- to eightfold increased resistance to penicillin without affecting response to other antimicrobial agents	7, 31, 32, 40, 103, 105, 106, 107
penB	Penicillin	*por*, *spc*	Nonspecific low-level resistance to penicillin, tetracycline, and nalidixic acid	24, 27, 40, 100, 103, 105, 106
pilA	Pilin	*pilE*	Gene product activates the *pilE* promoter in *E. coli*	117
pilB	Pilin	*pilE*	Gene product negatively regulates *pilE* promoter; insertional inactivation of *pilB* results in hyperpiliated gonococci	117
pilE	Pilin	*opa*[e]	Expressed locus for pilin structural gene	74, 75, 77, 113
pilS	Pilin	*opa*[e]	Silent (nonexpressed) locus for pilin structural gene	51, 74, 75, 113
por	Porin	*spc*, *penB*	*nmp*; outer membrane protein I structural gene	17, 20, 21, 45
proA	Proline	*proB*	Complementation of *proA* mutation of *E. coli* χ463	110
proB	Proline	*proA*	Complementation of *proB* mutation of *E. coli* χ340	110
proC	Proline		Complementation of *proC* mutation of *E. coli* χ278	H
pyrB	Pyrimidine		Aspartate transcarbamylase	99
pyrE	Pyrimidine		*pyr-2*; orotate phosphoribosyltransferase	99
recA	Recombination		Homologous recombination and DNA repair	63
rif	Rifampin	*ura*, *str*	Resistance to rifampin	5, 93
rmp	Reduction modifiable protein		Outer membrane protein III structural gene	42, 44
sac-1	Serum-antibody-complement	*penB*	Resistance to the bactericidal activity of normal human serum	18
sac-3	Serum-antibody-complement	*penB*	Resistance to the bactericidal activity of normal human serum; alteration in lipopolysaccharide structure	97, 112

Continued on following page

TABLE 2—*Continued*

Gene symbol	Mnemonic	Linkage association[a]	Alternative gene symbols, phenotypic trait affected	Reference(s)[b]
spc	Spectinomycin	*cap*, *penB*	High-level resistance to spectinomycin; alteration of 30S ribosomal subunit	70, 93
str	Streptomycin	*rif*, *fus*	High-level resistance to streptomycin; alteration of 30S ribosomal subunit	68, 70, 93
tem	Tetracycline modifier		Modifier gene affecting resistance to tetracycline and penicillin	122
tet	Tetracycline	*fus*, *cap*	Low-level resistance to tetracycline	68, 93, 105
trpE	Tryptophan		Complementation of *trpE* mutation of *E. coli* K-12 χ478	J
tsg	Temperature-sensitive growth		Family of uncharacterized mutations with the common phenotype of no growth or poor growth at 37°C	111, 125
ura	Uracil	*rif*	Requirement for uracil	5, 72
vel			Hypersusceptibility to vancomycin and erythromycin	60
vnc	Vancomycin		Hypersusceptibility to vancomycin	60

[a] Linkage associations refer to the linkage relationships diagrammed in Fig. 1 or to genes shown to be physically linked.

[b] Numbers refer to Literature Cited. The asterisk refers to a gene which has not yet been shown to exist in *N. gonorrhoeae*; however, researchers at the Sixth International Pathogenic Neisseria Meeting agreed that this nomenclature should be used. Letters refer to abstracts or personal communications from the listed individuals: (A) S. A. Berish, T. A. Mietzner, and S. A. Morse; (B) R. A. Jensen, R. K. Bhatnagar, and A. T. Hendry; (C) G. Biswas, S. Lacks, and P. F. Sparling; (D) R. Chien, A. Pierkarowicz, and D. Stein; (E) R. H. Chien, D. C. Stein, H. S. Seifert, K. Floyd, and M. So., Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, K41, p. 213; (F) P. R. Martin, D. A. Simpson, and M. H. Mulks; (G) E. F. Petricoin and D. C. Stein, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D187, p. 102; (H) F. Picard and J. R. Dillon; (I) F. Picard and J. R. Dillon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D173, p. 100; (J) D. C. Stein; (K) W.-M. Tsai and C. E. Wilde III; (L) J. P. Woods, S. M. Spinola, S. M. Strobel, and J. G. Cannon.

[c] We have assigned the mnemonic *dcm* to genes which encode a DNA cytosine methyltransferase and the mnemonic *der* to corresponding restriction endonuclease genes. The letters *A*, *B*, *C*, *D*, *E*, *G*, and *H* correspond to DNA sequences (methylase specificities) S.*Ngo*I, S.*Ngo*II, S.*Ngo*III, S.*Ngo*IV, S.*Ngo*V, S.*Ngo*VII, and S.*Ngo*VIII, respectively, recognized by a particular cytosine methyltransferase or restriction endonuclease as described by Davies (28).

[d] Cannon and Sparling (19) have designated this locus *nsr* to distinguish it from the phenotypically similar but genetically different locus *mtr*.

[e] In *N. gonorrhoeae* MS11$_{mk}$, three protein II loci, *opaE1*, *opaE2*, and *opaE3*, are adjacent to the unlinked pilin loci, *pilE1*, *pilS5*, and *pilS4*, respectively (74, 113). Schwalbe and Cannon (95) showed by genetic transformation that the six protein II loci in *N. gonorrhoeae* FA1090 are unlinked.

GENETIC MARKERS AND CLONED GENES OF *N. MENINGITIDIS*

Much less work has been done on identification of genetic markers in *N. meningitidis*. Genetic markers of *N. meningitidis* are listed in Table 5. As in *N. gonorrhoeae*, no standard laboratory strain has been used for the majority of these studies. *N. meningitidis* FAM18 (serogroup C, serotype 2A), isolated from the cerebrospinal fluid of a patient with meningococcal septicemia, was used to isolate the gene encoding the H.8 lipoprotein (*lip*) and to demonstrate amino acid and DNA homology between the class 5 outer membrane proteins of *N. meningitidis* and the proteins II of *N. gonorrhoeae* (56); it was also used to identify iron uptake mutants (33).

CONCLUSIONS AND PROSPECTS FOR THE FUTURE

A number of factors have been responsible for the inability to generate a comprehensive genetic map for *N. gonorrhoeae* or *N. meningitidis*. First and foremost is the small number of researchers involved in studies of these organisms as compared with those working with *E. coli*. Second, neither of these neisserial species contains the genetic manipulation systems, conjugation and transduction, that have enabled the interchange of large DNA fragments between strains. The only genetic system available, transformation (102), will demonstrate linkage only if genes are within about 30 to 40 kilobase pairs of each other (19).

A third factor limiting development of a useful genetic map is the difficulty in obtaining mutants. *N. gonorrhoeae* does not contain the error-prone repair systems, found in some other organisms (16), that allow the high-frequency generation of mutants in a mutagenized population. Mutagens such as ultraviolet light or methyl methanesulfonate kill *N. gonorrhoeae* without producing mutants in the surviving population. The effective mutagens, ethyl methanesulfonate and nitrosoguanidine, introduce lesions that result in mispairing and enhance the frequency of gonococcal mutants, but this enhancement occurs at a level significantly below that observed with other bacterial species. In addition to difficulties

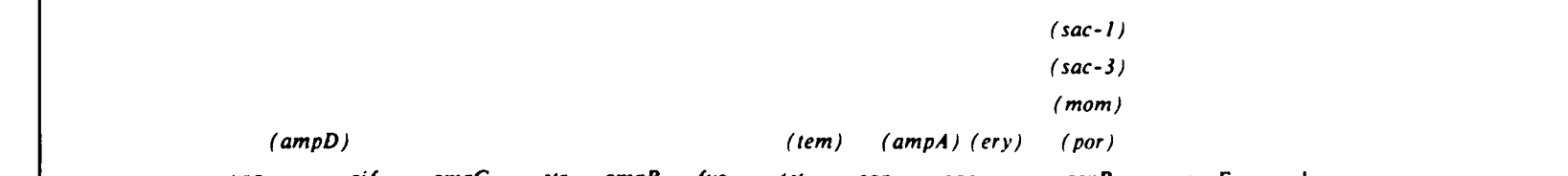

FIG. 1. Linkage relationships of chromosomal markers in *N. gonorrhoeae*. This map is based on mapping data obtained from several laboratories and with several strains of *N. gonorrhoeae* (5, 17–19, 55, 68, 69, 93, 97, 100, 103–105, 122, 126). Therefore, the distances shown do not correlate with actual genetic or physical distances on the gonococcal chromosome. Parentheses around a marker indicate an uncertainty about its position relative to adjacent markers. An explanation of the genetic symbols is given in Table 2.

TABLE 3. Cloned genes from *N. gonorrhoeae*

Gene symbol	Vector	*E. coli* host	*N. gonorrhoeae* parent	Selection or screening method	Reference(s)[a]
argA	λEMBL4	NM539	CH811	Lytic complementation of *argA* mutation of *E. coli* NK5992 and W3421	G
argB	λEMBL4	NM539	CH811	Lytic complementation of *argB* mutation of *E. coli* 30SOMA4	G
argE	λEMBL4	NM539	CH811	Lytic complementation of *argE* mutation of *E. coli* AB1157	G
argE	pUC12	AT2538	CDC50	Complementation of *argE* mutation of *E. coli* AT2538	D
argF	λEMBL4	NM539	CH811	Lytic complementation of *argI* mutation of *E. coli* N166	G
argF	pUC12	N134	CDC50	Complementation of *argF argI* mutation of *E. coli* N134	D
argG	λEMBL4	NM539	CH811	Lytic complementation of *argG* mutation of *E. coli* UQ27	G
carA	λEMBL4	NM539	CH811	Lytic complementation of *carA* mutation of *E. coli* CP178	G
carB	λEMBL4	NM539	CH811	Lytic complementation of *carB* mutation of *E. coli* HF8	G
dcmA	pBR322	RR1	P9	Resistance of recombinant plasmids to digestion with *Hae* II	114
dcmB	pBR322	RR1	P9	Resistance of recombinant plasmids to digestion with *Hae* III	114
dcmD	pHSS7	GC6	MS11	Resistance of recombinant plasmid to digestion with *Nae* I	C
dcmE	pHC79	HB101	MUG116	Resistance of recombinant plasmids to digestion with *Bam*HI, *Kpn*I, and *Nar*I	B, H
dcrD	pHSS7	GC6	MS11	Resistance of recombinant plasmids to digestion with *Nae*I	C
dcrE	pHC79	HB101	MUG116	Resistance of recombinant plasmids to digestion with *Bam*HI, *Kpn*I, and *Nar*I	B
fbp	pUC13 pUC19	JM105	F62	Hybridization with *fbp*-specific synthetic oligonucleotide probes	A
fhuB	λSE4	RR1	FA19	Complementation of *fhuB* mutation of *E. coli* BU736 and BN3307	124
iga	pREG152	HB101	F62	Cleavage of human immunoglobulin A as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis	61
iga	pBR322	GC1	MS11	Filter assay using radiolabeled human immunoglobulin A antibody bound to nitrocellulose via its secretory component	52
laz	λgt11	Y1089	R10	Immunoblotting with anti-protein I antiserum	42, 43
lip	λsep6	LE392	FA1090	Immunoblotting with monoclonal antibody H.8	12
lps-1	pBR322	HB101	RUN4383	Colony immunoblotting with antigonococcal outer membrane antiserum	86
lps-2	pHC79	HB101	MUG116	Complementation of pyocin-resistant phenotype of *N. gonorrhoeae* FA5100	E
lps-3	pHC79	HB101	MUG116	Complementation of pyocin-resistant phenotype of *N. gonorrhoeae* FA5100	E
metB	pLES2	χ342	JW31	Complementation of *metB* mutation of *E. coli* χ342	89
nal	pHC79	HB101	MUG116	Transformation of cosmid library into *N. gonorrhoeae* FA5100 with selection for nalidixic acid resistance	H, I
omc	λgt11 and λEMBL3	Y1090 and Y1088	2686	Immunoblotting with antigonococcal outer membrane protein-macromolecular complex antiserum	J
opa	pBR322	GC1[b]	$MS11_{ms}$	Colony immunoblotting with anti-MS11 protein II antiserum	113
opa	pBR322	HB101	FA1090	Colony radioimmunoassay with monoclonal antibodies specific for FA1090 protein II variants	26
oxiA	pBR322	HB101	RUN4007	Colony immunoblotting with antigonococcal outer membrane antiserum	36, 37
penA	pBG5131	TG1	CDC77-124615	Transformation of *N. gonorrhoeae* FA19 to increased penicillin resistance (sixfold)	107
penA	pBG5131	TG1	LM306, FA19, CDC84-060418, and CDC84-060384	Hybridization with *penA* gene from CDC77-124615	107
pilA	pBR322	GC1	$MS11_{ms}$	Characterization of previously isolated clones containing *pilE* and *opaE1*	117
pilB	pBR322	GC1	$MS11_{ms}$	Characterization of previously isolated clones containing *pilE* and *opaE1*	117
pilE1	pBR322	GC1	$MS11_{ms}$	Colony immunoblotting with anti-pilin antiserum	77
pilE	pBR322	GC1	P9	Colony immunoblotting with anti-pilin monoclonal antibody SM1	87
por	pGEM-2	HB101	FA19	Hybridization with protein 1A-specific synthetic oligonucleotide probes	21
por	pGEM-3 and λgt11	HB101	MS11	Hybridization with protein I-specific synthetic oligonucleotide probes	20
por	λgt11	Y1089	R10	Immunoblotting with anti-R10 protein 1B monoclonal antibodies	45
proA	pLES2	JM83	KH45	Complementation of *proAB* mutation of *E. coli* JM83	110
proB	pLES2	JM83	KH45	Complementation of *proAB* mutation of *E. coli* JM83	110

Continued on following page

TABLE 3—*Continued*

Gene symbol	Vector	*E. coli* host	*N. gonorrhoeae* parent	Selection or screening method	Reference(s)[a]
proC	pGEM-3	χ478	CH811	Complementation of *proC* mutation of *E. coli* χ478	F
rmp	λgt11	Y1089	R10	Immunoblotting with polyclonal anti-protein I antiserum	42
sac-4	pHC79	HB101	JC1	Transformation of *N. gonorrhoeae* F6. to serum resistance	73
recA	pREG153	HB101	$MS11_{mk}$	Complementation of *recA* mutation of *E. coli* HB101	63
rif	pHC79	HB101	MUG116	Transformation of cosmid library into *N. gonorrhoeae* FA5100 with selection for rifampin resistance	H
trpE	pLES2	JM83	JW31	Complementation of *trpE* mutation of *E. coli* K-12 χ478	H

[a] Numbers refer to Literature Cited. Letters refer to abstracts or personal communications from the listed individuals: (A) S. A. Berish, T. A. Mietzner, and S. A. Morse; (B) R. Chien, A. Piekarowicz, and D. Stein; (C) R. H. Chien, D. C. Stein, H. S. Seifert, K. Floyd, and M. So, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, K41, p. 213; (D) P. R. Martin, D. A. Simpson, and M. H. Mulks; (E) E. F. Petricoin and D. C. Stein, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D187, p. 102; (F) F. Picard and J. R. Dillon; (G) F. Picard and J. R. Dillon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D173, p. 100; (H) D. C. Stein; (I) D. C. Stein and T. Cook; (J) W.-M. Tsai and C. E. Wilde III.

[b] *E. coli* GC1 is (K-12$_{\lambda^-}$) (52, 77).

[c] Additional expressed and silent pilin genes have been cloned from strains MS11 (51, 75) and P9 (83).

[d] We have designated the serum resistance allele of *N. gonorrhoeae* JC1 *sac-4*. This allele may be identical to the previously described *sac-3* allele in *N. gonorrhoeae* F62 (97).

TABLE 4. Codon usage in *N. gonorrhoeae*[a]

Amino acid	Codon[b]	No.[c]	No. 1,000[d]	Fraction[e]	Amino acid	Codon	No.	No. 1,000	Fraction
Gly	GGG	31	6.34	0.07	Trp	TGG	52	10.63	1.00
Gly	GGA	55	11.24	0.13	End	TGA	10	2.04	0.50
Gly	GGT	103	21.05	0.25	Cys	TGT	8	1.63	0.20
Gly	GGC	228	46.6	0.55	Cys	TGC	32	6.54	0.80
Glu	GAG	40	8.17	0.18	End	TAG	1	0.2	0.05
Glu	GAA	187	38.22	0.82	End	TAA	9	1.84	0.45
Asp	GAT	121	24.73	0.44	Tyr	TAT	87	17.78	0.51
Asp	GAC	152	31.06	0.56	Tyr	TAC	82	16.76	0.49
Val	GTG	54	11.04	0.17	Leu	TTT	106	21.66	0.30
Val	GTA	67	13.69	0.21	Leu	TTA	49	10.01	0.14
Val	GTT	104	21.25	0.33	Phe	TTT	76	15.53	0.45
Val	GTC	93	19.01	0.29	Phe	TTC	94	19.21	0.55
Ala	GCG	82	16.76	0.17	Ser	TCG	33	6.74	0.10
Ala	GCA	131	26.77	0.27	Ser	TCA	54	11.04	0.16
Ala	GCT	64	13.08	0.13	Ser	TCT	85	17.37	0.25
Ala	GCC	205	41.90	0.43	Ser	TCC	76	15.53	0.22
Arg	AGG	23	4.70	0.07	Arg	CGG	49	10.01	0.16
Arg	AGA	28	5.72	0.09	Arg	CGA	29	5.93	0.09
Ser	AGT	21	4.29	0.06	Arg	CGT	67	13.69	0.22
Ser	AGC	73	14.92	0.21	Arg	CGC	112	22.89	0.36
Lys	AAG	58	11.85	0.15	Gln	CAG	78	15.94	0.35
Lys	AAA	331	67.65	0.85	Gln	CAA	146	29.84	0.65
Asn	AAT	106	21.66	0.37	His	CAT	51	10.42	0.45
Asn	AAC	177	36.17	0.63	His	CAC	63	12.88	0.55
Met	ATG	60	12.26	1.00	Leu	CTG	78	15.94	0.22
Ile	ATA	27	5.52	0.15	Leu	CTA	25	5.11	0.07
Ile	ATT	67	13.69	0.36	Leu	CTT	62	12.67	0.17
Ile	ATC	90	18.39	0.49	Leu	CTC	38	7.77	0.11
Thr	ACG	53	10.83	0.20	Pro	CCG	74	15.12	0.37
Thr	ACA	48	9.81	0.18	Pro	CCA	35	7.15	0.17
Thr	ACT	45	9.20	0.17	Pro	CCT	44	8.99	0.22
Thr	ACC	117	23.91	0.44	Pro	CCC	47	9.61	0.23

[a] The program CodonFrequency from the University of Wisconsin Genetics Computer Group (30) was used to generate codon usage tables from each of the *N. gonorrhoeae* genes in the GenBank (release 56, 7/88) and EMBL (release 15, 4/88) data bases (10, 15). A reference codon usage table was generated from the individual codon usage tables for the following genes: *pilE1* and *pilS1* from $MS11_{ms}$ (51, 75); three protein II genes from FA1090 (26); the protein I gene from FA19 (21); *penA* from CDC77-124615 (107); the gene for an azurin-related protein from MS11 (43); the gene for protein III from R10 (44); the immunoglobulin A protease gene from $MS11_{ms}$ (90); and the M.*Ngo*PII DNA methyltransferase gene from P9 (115).

[b] Total number of codons, 4,893.

[c] The number of times a codon occurred in the examined sequences.

[d] The number of times a specific codon would occur per 1,000 codons.

[e] The ratio of the number of occurrences of a specific codon to the number of occurrences of all codons in the same synonymous codon group.

in obtaining mutants, *N. gonorrhoeae* does not contain a variety of genes that could be mutated that are not critical to the growth of the organism, such as the variety of genes involved in catabolism of alternative fuel sources that are found in *E. coli*. Thus, there are fewer mutations available to serve as genetic markers in mapping studies in *N. gonorrhoeae*.

The advent of recombinant DNA techniques has been invaluable to the studies of genes in both *N. gonorrhoeae* and *N. meningitidis*; these techniques have proved to be a useful alternative to classical genetic techniques. We expect that the isolation of neisserial genes will continue and will greatly expand the number of identified genes available for linkage studies. New technologies are continuing to become available, and the one that may prove the most useful in studies of the genetic organization of *N. gonorrhoeae* and *N. meningitidis* is pulsed-field electrophoresis. The discovery of restriction enzymes that recognize 8-base-pair sequences and the ability to separate very large DNA fragments by altering the orientation of the electric field during agarose gel electrophoresis has enabled researchers to physically map the *E. coli* chromosome (101). It should also be possible to generate physical maps of the gonococcal and meningococcal chromosomes by this technique, especially since the neisserial chromosomes are only about 50% of the size of the *E. coli* chromosome (19). Once such maps are developed, the position of a cloned gene on the map could be easily determined by DNA-DNA hybridization.

If physical maps are developed for *N. gonorrhoeae*, we strongly suggest that they be done with one or more of the gonococcal strains that are the most frequently studied (e.g., see Table 1). We also urge investigators to use one of these strains as the source of DNA for their cloning experiments, whenever possible. This would allow mapping of the location of the cloned gene to one or more of the fragments whose position is known on the physical map. Both *N.*

TABLE 5. Genetic markers of *N. meningitidis*

Gene symbol	Mnemonic	Phenotypic trait affected or cloned gene	Cloning or mutagenesis strategy	Reference(s)[a]
cps-1	Capsule	Biosynthesis and assembly of group B polysaccharide capsule	pCOS2EMB1, library of *N. meningitidis* B1940 in *E. coli* GC6 screened with monoclonal antibody	76
cps-2	Capsule	5′-Monophospho-*N*-acetyl neuraminic acid synthetase	pACYC184 library of *N. meningitidis* group B was screened by complementation of the corresponding mutation in *E. coli* EV5	D
cps-3	Capsule	*N*-Acetylneuraminic acid synthetase	pACYC184 library of *N. meningitidis* group B was screened by complementation of the corresponding mutation in *E. coli* EV5	D
fbp	Iron-binding protein	Iron-binding outer membrane protein (37,000 daltons); detected by immunoblotting with gonococcal anti-*fbp* antisera		80
frp	Iron-repressible protein	Family of outer membrane proteins expressed under conditions of iron starvation		11, 33, 34
fud-2	Iron uptake deficient	Deficient in iron acquisition from transferrin	Ethyl methanesulfonate mutagenesis of FAM20 (a Nal^r derivative of FAM18) followed by streptonigrin enrichment	33, 120
fud-3	Iron uptake deficient	Deficient in iron acquisition from transferrin and hemoglobin	Ethyl methanesulfonate mutagenesis of FAM20 followed by streptonigrin enrichment	33
fud-4	Iron uptake deficient	Deficient in iron acquisition from ferric dicitrate, transferrin, lactoferrin, hemin, and hemoglobin	Streptonigrin enrichment of log-phase culture of FAM20	33
fud-5	Iron uptake deficient	Deficient in iron acquisition from ferric dicitrate, transferrin, lactoferrin, and hemin	Streptonigrin enrichment of log-phase culture of FAM20	33
hga	Hemoglobin growth alteration	Altered ability to grow with hemoglobin as sole iron source	Streptonigrin enrichment of log-phase culture of FAM20	33, 120
iga	Immunoglobulin A	Immunoglobulin A protease	pBR325 library of *N. meningitidis* 15894 in *E. coli* HB101 screened by hybridization with gonococcal immunoglobulin A protease probe	62
laz	Lipid-modified azurin	Azurin-related outer membrane protein	pBR322 library of *N. meningitidis* FAM18 in *E. coli* HB101 screened by colony immunoblotting with monoclonal antibody H.8	57
omc	Outer membrane complex	Structural gene for *N. gonorrhoeae omc* hybridizes to *N. meningitidis* DNA		E
opa	Opacity-associated protein	Class 5 outer membrane protein; shares homology with *N. gonorrhoeae opa* including pentameric repeat	pBR325 library of *N. meningitidis* FAM18 in *E. coli* HB101 screened by colony blot radioimmunoassay with monoclonal antibodies H.21 and H.22	41, 56
oxiA	Oxygen induced	Structural gene for *N. gonorrhoeae oxiA* hybridizes to *N. meningitidis* DNA and anti-OxiA antiserum reacts with a meningococcal protein of the same size		37
pilE	Pilin	Expressed class I pilin locus	pBR322 library of *N. meningitidis* C311 in *E. coli* GC1 screened by hybridization with oligonucleotide to the coding sequence of the SM1 epitope of gonococcal pilin	91
pilS	Pilin	Silent (nonexpressed) class II pilin locus	pBR322 library of *N. meningitidis* C114 in *E. coli* GC1 screened by hybridization with *pilE* gene of *N. gonorrhoeae* P9-2	88
porA	Porin	Class 1 outer membrane protein structural gene	λgt11 library of *N. meningitidis* MC50 in *E. coli* Y1090 screened by immunoblotting with antiserum against purified outer membranes	8, 41, A
porB	Porin	Class 2 and 3 outer membrane proteins; shares DNA homology with *N. gonorrhoeae por*; antibody to gonococcal protein IB cross-reacts with class 2 proteins	λgt11 library of group B *N. meningitidis* screened with a monoclonal antibody against *N. gonorrhoeae* protein IB and by hybridization with a synthetic oligonucleotide	41, C
rmp	Reduction-modifiable protein	Class 4 outer membrane protein; shares DNA homology with *N. gonorrhoeae rmp*; antibody to gonococcal protein III cross-reacts with class 4 proteins	λgt11 library of *N. meningitidis* BNCV was screened by immunoblotting with a monoclonal antibody to gonococcal protein III	41, B

[a] Numbers refer to Literature Cited. Letters refer to personal communications from the following individuals: (A) A. K. Barlow, J. E. Heckels, and I. N. Clarke; (B) K. P. Klugman and E. C. Gotschlich; (C) K. Murakami and E. C. Gotschlich; (D) C. Reid, S. Ganguli, T. Wallis, I. Roberts, and G. Boulnois; (E) C. E. Wilde III.

gonorrhoeae and *N. meningitidis* have been shown by restriction length polymorphism studies to have a high degree of sequence variability (39, 64). Thus, it is anticipated that the physical map determined by using pulsed-field electrophoresis of large fragments will be different for every strain. The use of only a few strains in developing these maps and in cloning experiments will allow researchers from different laboratories to compare their data much more readily and thus enable a more rapid analysis of neisserial gene organization. We further suggest that the above-mentioned strains be deposited into the American Type Culture Collection and the National Collection of Type Cultures to make them more readily available to the general research community.

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Gonococcal and Meningococcal Pathogenesis as Defined by Human Cell, Cell Culture, and Organ Culture Assays

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Neisseria gonorrhoeae and *N. meningitidis* are exclusive human pathogens. This important fact has limited the use and the relevance of animal models (30) in studies of the pathogenesis of these organsims. The potential severity of infections caused by gonococci and meningococci has limited direct human experimentation. Only studies of gonococcal urethritis in men (29, 63) and testing of potential vaccine candidates have been possible. Human tissue specimens obtained from infected individuals has provided important information (12, 14, 22), but these specimens are limited in availability and are often obtained late in the course of infection.

To address these problems, in vitro cell and organ culture assays involving predominantly cells and tissues of human origin have been developed. These assays have been used to identify important mechanisms by which gonococci and meningococci cause disease. They have provided much of the experimental data about three major pathogenic events: (i) mucosal cytotoxicity, (ii) attachment of gonococci and meningococci to epithelial cells, and (iii) mucosal invasion. This review will focus on the data derived from the use of human cells, cell lines, and organ cultures in defining these events.

DESCRIPTION OF HUMAN CELLS, CELL LINES, AND ORGAN CULTURES

The following human cells and cultures are used in studies of gonococcal and meningococcal pathogenesis: isolated buccal, pharyngeal, endocervical, vaginal, urethral, sperm, and fetal tonsil cells; cervical carcinoma (HeLa), Chang conjunctival epithelial, endometrial carcinoma (HEC-I-B, ENCA-4), and larynx carcinoma (HEp-2) cell lines; primary embryonic lung, fibroblast, amnion, endometrial, endocervical, and foreskin cell cultures; and fallopian tube, nasopharyngeal, and ectocervical organ cultures. Animal cells, cell lines, and organ cultures have also been used (1, 20, 35). However, when they are compared with human tissue from the same site, major differences are reported. For example, gonococci attach to nonciliated mucosal cells of the human fallopian tube and damage the ciliary function of human fallopian tube organ cultures but not fallopian tube organ cultures of rabbits, pigs, and cows (17, 27, 28).

Human buccal epithelial cells have been favored for use in attachment assays and are easily obtained (Fig. 1). Buccal cells are squamous epithelial cells obtained by scraping the buccal mucosa. Gonococci and meningococci attach to but do not invade these cells. However, the availability of buccal cells is offset by several factors. Cells obtained are not uniform in the degree of maturation, viability, and size, and their structure is influenced by hormonal factors and other variables that are not easily controlled. In addition, many of these cells already have bacteria (oral flora) and variable amounts of mucus attached to their surfaces. Furthermore, the buccal mucosa is not normally colonized by pathogenic *Neisseria* species. Despite these drawbacks, buccal cells have been extensively used in studies of gonococcal and meningococcal attachment (23, 44, 45, 56, 58, 61, 62, 64, 65).

Isolated squamous epithelial cells from mucosal surfaces closer to natural sites of meningococcal and gonococcal colonization (e.g., vaginal, ectocervical, urethral, oropharyngeal, and tonsillar sites) have also been used. Although potentially of more relevance, these isolated cells have similar disadvantages to those described for buccal cells. Human erythrocytes of various blood groups (ABO-Rh) are also a favorite cell type for attachment assays. Sterility, availability, and uniformity of size are advantages. Hemagglutination assays are most commonly used for attachment studies. Recently a hemadsorption assay on nitrocellulose disks (16) also has been used to more precisely define the phenotypes of meningococci which bind to erythrocytes. Caution must be exercised when observations made with erythrocytes are generalized to interactions with mucosal cells. For example, Lambden et al. (31) noted increased binding of protein II (PII)-containing gonococci to buccal cells but not to erythrocytes. Gonococci that did not express PII exhibited increased binding to erythrocytes.

Primary epithelial cell lines (e.g., uterine, endometrial, conjunctival, and ectocervical cells) overcome many of the disadvantages of isolated cells but are often difficult to establish and maintain. Cell lines, often of tumor origin, are less fragile and have been extensively used. HeLa cells (a human cervical carcinoma cell line), HEp-2 cells (a human laryngeal carcinoma cell line), human endometrial carcinoma cell lines, and Chang conjunctival epithelial cells have been the most widely used (see above) (2, 4, 21, 52, 66). These cell lines have been used for cytotoxicity and invasion studies as well as attachment assays.

Infection of organ cultures has provided an experimental means of studying the interactions of gonococci and meningococci with intact mucosal surfaces (10, 26, 36, 38, 53, 70). Human fallopian tube organ cultures (FTOC) are used because they are a site of natural infection (gonococcal salpingitis). Infections in this model produce changes similar to those noted in pathologic specimens obtained during salpingitis. Human nasopharyngeal tissue in organ culture (NPOC) has been used to study *N. meningitidis* (53). The specificity of the events observed in these models and their correlation with events observed during natural infection are major advantages. Difficulty in obtaining these tissues, variability among tissue from different individuals, and absence of important components of the inflammatory response (e.g., leukocytes, circulating antibody, and complement) are disadvantages. In addition, they require antibiotics for sterilization and have limited viability. Nevertheless, human organ culture assays have provided valuable clues about mechanisms of gonococcal and meningococcal cytotoxicity, attachment, and invasion.

PATHOGENESIS

Contact of gonococci or meningococci with human mucosal surfaces (e.g., urogenital tract for *N. gonorrhoeae*, nasopharynx for *N. meningitidis*) initiates a series of events resulting in attachment of the organisms to epithelial cells and multiplication (colonization) at the mucosal surface. These events may also lead to internalization of gonococci or meningococci by epithelial cells and possibly transport of the organisms across the normally protective mucosal barrier. Clinically this process may result in asymptomatic gonococcal or meningococcal carriage, or in signs of inflammation, indicating injury to host cells. For example, most individuals who harbor meningococci or gonococci in the nasopharynx are asymptomatic. Overt pharyngitis resulting from infection by either organism is unusual. In contrast, infection of the human urethra or human fallopian tube by gonococci often results in marked inflammation and tissue damage. Table 1 is a summary of pathogenic events noted following gonococcal or meningococcal infection of isolated human cell, cell culture, and organ culture assays.

Mucosal Cytotoxicity

Many of the clinical signs of gonococcal or meningococcal infection are due to migration of leukocytes and activation of complement at the site of infection. However, there is increasing evidence that gonococci and meningococci exert direct cytotoxic effects that may potentiate the inflammatory response. In contrast, commensal *Neisseria* strains do not normally cause overt cytotoxicity. By using the cell and organ culture experimental models described above, the mechanisms by which *N. gonorrhoeae* and *N. meningitidis* may directly induce cytopathic changes at mucosal surfaces have been partially determined.

Virji et al. studied the cytotoxic effect of gonococci on Chang epithelial cells (66–68). Variants of *N. gonorrhoeae* P9 that expressed pili or PII were cytotoxic, whereas gonococci that did not express these components were not. Pili and PII also increased the attachment of the gonococci to this cell line. These data suggest that close, direct contact facilitated by attachment ligands may be important in local cytotoxicity.

FIG. 1. Scanning electron micrograph showing attachment of piliated meningococci to human buccal epithelial cells. Meningococci attach to microplicae of these cells, usually in areas free of mucus.

Damage to ciliary activity was noted early in the course of

TABLE 1. Events noted with gonococcal or meningococcal infection of human cell, cell culture, and organ culture assays

Cell or organ type and infection	Attachment[a]	Cytotoxicity[a]	Invasion[a]
N. gonorrhoeae			
Buccal epithelial cells	$P^+ > P^-$, $PII^+ > PII^-$	N	N
Cervical epithelial cells	$P^+ > P^-$, $PII^+ > PII^-$	N	N
Erythrocytes	$P^+ > P^-$, $PII^- > PII^+$	N(?)	N
HeLa cells	$PII^+ > PII^-$	N	$PII^+ = PII^-$, $P^+ = P^-$
Chang conjunctival cells	$PII^+ > PII^-$	Y	Y
HEC-I-B cells	Y	Y	$P^+ = P^-$
HEp-2 cells	Y	N(?)	Y
FTOC	$P^+ > P^-$, $PII^+ > PII^-$ (?)	$P^+ > P^-$	$P^+ = P^-$
N. meningitidis			
Buccal epithelial cells	$P^+ > P^-$	N	N
Erythrocytes	$P^+ > P^-$	N(?)	N
NPOC	$P^+ > P^-$	Y	Y
Commensal *Neisseria* spp.			
Buccal epithelial cells	Y	N	N
Chang conjunctival cells (*N. mucosa*)	Y		N
HEC-I-B cells (*N. lactamica*)	Y	N	N
HeLa cells (*N. sicca*)	Y	N	N
NPOC (*N. subflava*)		N	
NPOC (*N. lactamica*)	Y		Y

[a] Abbreviations: Y, observed; N, not observed; P^+, piliated; P^-, nonpiliated; PII^+, expressing II; PII^-, not expressing PII.

FIG. 2. Scanning electron micrograph showing attachment of meningococci to nonciliated columnar epithelial cells but not ciliated cells of human nasopharyngeal organ cultures. Note the general loss of short microvilli on nonciliated cells to which meningococci attach. They are replaced by a few elongated microvilli (arrows) interacting with attaching meningococci.

human FTOC infection with gonococci and human NPOC infection with meningococci (55). Loss of ciliary activity was accompanied by sloughing of ciliated cells. Unlike *Bordetella pertussis*, which damages ciliated cells after direct attachment (15), the damage to the ciliated cells was not associated with the attachment of gonococci or meningococci to these cells (Fig. 2) or to the presence of organisms within ciliated cells. Infection with the commensal species *N. subflava* did not result in significant damage to human FTOC or NPOC ciliary activity. Lipopolysaccharide (LPS) appears to be a major toxin of gonococci for the ciliated cells of human FTOC (18, 19, 36, 41). Gonococcal peptidoglycan fragments also damage FTOC ciliary activity (42). Both piliated and nonpiliated gonococci and meningococci damage FTOC and NPOC ciliary activity (36, 39, 57), but piliated organisms damage ciliary activity more rapidly than nonpiliated organisms do. Ciliated cells of the FTOC were damaged by <10 μg of purified gonococcal or meningococcal LPS per ml (19). By 1 to 2 h after exposure to gonococcal LPS, vesicles containing LPS were distributed throughout the cytoplasm of ciliated cells (9). Polymyxin B neutralized LPS-induced damage, suggesting that the lipid A portion of LPS was the toxic moiety. In contrast, purified gonococcal and meningococcal LPS at 100 μg/ml did not damage NPOC from humans (57) or FTOC from rabbits, pigs, and cows (17). These studies indicate that *N. gonorrhoeae* and possibly *N. meningitidis* damage ciliated epithelial cells indirectly by release of LPS, peptidoglycan monomers, and possibly other toxins from the organisms and suggest that there are differences in the susceptibility of ciliated cells to these toxins. The selectivity of the LPS toxicity for humans and for specific human mucosal surfaces may be responsible in part for the host specificity of infections and the variability in severity of human mucosal infections due to gonococci or meningococci.

Pili are not necessary for damage to ciliated cells (39, 55, 57) but facilitate the attachment of meningococci and gonococci to nonciliated epithelial cells of both mucosal surfaces (39, 56). The increased attachment associated with pili may allow more effective delivery of toxic factors to adjacent ciliated epithelial cells. Other outer membrane proteins of gonococci or meningococci did not appear to be required for damage to ciliated cells (55) but detailed studies to address this question have not been performed. Immunoglobulin A1 protease activity was also not critical for epithelial cell damage seen in gonococcal infection of FTOC (7, 8). Similar results have been noted when immunoglobulin A1 protease activity-deficient mutants of *Haemophilus influenzae* were used to infect NPOC (13). However, in view of recent studies defining other products of the immunoglobulin A1 protease gene (J. Pohlner and T. Meyer, personal communication) which may affect host cells and the finding of other substrates cleaved by this protease, these studies must be viewed cautiously.

Attachment

Although nonspecific factors (e.g., surface charge, pH, Derjaguin-Landau-Verwey-Overbeek [DLVO] theory, ionic bridging, and hydrophobic interactions) may be important (72), attachment of gonococci and meningococci to human cells is selective. In the FTOC and NPOC models (7, 10, 39, 53), gonococci and meningococci attach only to microvilli of nonciliated columnar epithelial cells. Attachment to ciliated cells is not observed. Similarly, attachment of piliated meningococci differs markedly among epithelial cells from different sites (54). In contrast, nonpiliated meningococci attach equally but in small numbers to all cell types. These data suggest that gonococcal and meningococcal attachment is mediated by specific ligands that selectively recognize receptors on certain types of human cells. The number and distribution of receptor sites for these ligands may in part determine sites of mucosal colonization and infection. As reviewed by Brooks (5), the host factors important in attachment of gonococci are poorly understood. The characteristics of the receptors for gonococci or meningococci and the influence of hormonal factors are currently areas of intense investigative interest.

Studies with human cells, cell lines and organ cultures have implicated three gonococcal and meningococcal surface components in attachment: (i) pili, (ii) heat-modifiable outer membrane proteins (e.g., gonococcal protein II), and (iii) the major porin proteins. However, it is critical to note that different mechanisms are probably operative in the attachment of gonococci and meningococci to different kinds of cells.

Pili are hairlike surface appendages which radiate several thousand nanometers from the surfaces of meningococci and gonococci. Numerous studies (1, 4, 6, 60, 64–67) with a variety of cells and organ cultures have implicated pili as major attachment ligands of meningococci and gonococci. Experiments with human volunteers have confirmed these findings (29, 63). Owing to their radiation from the cell

surface, these organelles probably make the initial contact with host cells. There is experimental evidence that supports this concept (70).

No clear differences in the degree of attachment were reported when different antigenic types of gonococcal pili were used in assays of attachment to buccal epithelial cells or to erythrocytes (50). In contrast, Trust et al. (65) noted that although all piliated meningococci attached to buccal cells, only certain strains bound to erythrocytes. In studies with antisera raised to purified pili, attachment and virulence for Chang conjunctival epithelial cells were reduced only in the presence of homologous antisera to pili (67). Heterologous antisera to pili were largely ineffective in reducing the attachment and cytotoxicity to these cells. Similar results are reported for attachment of gonococci to buccal epithelial cells (64) and rhesus monkey kidney cells (1). Schoolnik et al. (49) noted that a ^{125}I-labeled gonococcal pilin fragment, TC-2, bound to endocervical cells from healthy women but not to buccal or HeLa cells. Rothbard et al. (48) found that antibodies to synthetic peptides 69-84 and 41-50 inhibited a heterologous gonococcal strain from binding to the human endometrial carcinoma cell line ENCA-4. However, these antibodies were not effective in blocking attachment of meningococci to buccal epithelial cells, even though the epitopes were present on pilins of these strains (58). Thus, pili are important in the attachment of meningococci and gonococci to a variety of human cells, and they appear to mediate attachment through several different mechanisms.

For gonococci, PII also appears to be an important attachment ligand, at least to certain kinds of human cells. Lambden et al. (31) found that gonococci that expressed PII (regardless of the molecular weight of the PII) attached in greater numbers to buccal epithelial cells. Elkins et al. (11) noted that gonococci expressing PII showed increased adherence to primary cultures of uterine and ectocervical cells. Bessen and Gotschlich studied PII binding to HeLa cells (2) and noted that the receptor had a protein configuration (3). Lammel et al. (32) noted that a monoclonal antibody directed at a gonococcal PII decreased the adherence of gonococci expressing the PII to HEC-1-B cells. Similar results were reported by Sugasawara et al. for HeLa cells (59). PIIs are also important in binding to human polymorphonuclear leukocytes (11). In contrast, James et al. (25) and Draper et al. (10) found that transparent gonococci which lacked PIIs appeared to have increased attachment to FTOC and cervical explant tissue. Lambden et al. (31) also noted that in contrast to buccal cells, gonococci lacking PIIs demonstrated the greatest binding to erythrocytes. Gonococci lacking PIIs are often recovered from the fallopian tubes of women with salpingitis. These data indicate that PII may be important in attachment to certain kinds of human cells and a disadvantage in attachment to others. The question remains, however, an open one. Recently, Lammel et al. (C. J. Lammel, N. P. Dekken, and G. F. Brooks, personal communication) studied the ability of four PII-expressing gonococcal clones and a PII-negative clone to attach to human fallopian tube tissue. Differences in attachment and damage to FTOC mucosal cells occurred with different PII-expressing clones ($PII^+ > PII^-$).

Meningococci express heat-modifiable surface proteins (class V proteins) that are similar biochemically to PII. The role of these proteins in meningococcal attachment is much less clear. We noted (56) that meningococci that formed opaque colonies exhibited increased attachment to buccal epithelial cells, but we could not establish a relationship between colony phenotype and the expression of class V proteins. Others have observed (M. Hagman, P. Olan, and D. Danielsson, personal communication) that meningococci isolated from urogenital specimens and containing class V proteins showed an increased attachment to human vaginal cells but not to buccal epithelial cells.

As discussed in the next section, the major porin proteins of gonococci and meningococci have been shown to insert into eucaryotic membranes. These proteins may further enhance gonococcal attachment and initiate invasion.

Mucosal Invasion

Invasion of mucosal surfaces by *N. gonorrhoeae* has been noted histopathologically since the late 1800s. Harkness (22) reviewed data showing that in patients with acute gonorrhea, gonococci had penetrated the mucosal surface and were multiplying in the subepithelial space by day 3 of infection. However, it is unclear whether squamous cells of the cervix are truly invaded by gonococci (12). Intracellular gonococci are observed in histopathological specimens within nonciliated columnar epithelial cells of the urethra, cervix, and fallopian tubes (71, 72). Human fallopian tube organ cultures have been a major experimental model in the study of this event (38, 40, 70). Recently, a similar invasion event has been noted in human nasopharyngeal organ cultures infected with *N. meningitidis* (53). Shaw et al. (51, 52) developed an assay to study gonococcal invasion by using HEC-1-B human endometrial cells; Bessen and Gotschlich (2) and Richardson and Sadoff (46) studied this event in HeLa or human amnionic cells from primary cultures. Chang conjunctival epithelial cells (23, 67) and HEp-2 cells (4) have also been used to study gonococcal invasion, as have a variety of other mammalian cell cultures (4, 43, 69).

Several investigative groups have used the human fallopian tube model to study gonococcal mucosal invasion (40, 70). Approximately 1% of attached gonococci invade by 8 h after infection in this model. Attachment of gonococci to nonciliated mucosal cells is the first step in a process of internalization of gonococci by these cells. Similar to the relationship of cytotoxicity and attachment, attachment and invasion are closely associated. Virji et al. found increased cytotoxicity and invasion of Chang conjunctival cells by gonococci that exhibited the greatest attachment (67, 68).

After attachment of gonococci to nonciliated epithelial cells of the human fallopian tube, the microvilli of these cells surround gonococci and draw them to the surface of the mucosal cell. Later in the course of infection, the membranes of some of the nonciliated cells seem to retract and pinch off a membrane-bound vacuole containing gonococci. Similar membrane-bound vacuoles containing gonococci are noted following gonococcal infection of tissue culture cells (4, 43, 69). This process is quite similar to the phagocytosis of bacteria by professional phagocytes, but destruction of gonococci after entry into phagocytic vacuoles is not observed. Gonococci are rapidly transported in these vacuoles across the epithelial cell. In the FTOC model there is an orderly parting of the basement membrane, with release of gonococci into the subepithelial space.

In the NPOC model, similar events are observed following meningococcal infection (53). However, after meningococci enter via phagocytic vacuoles, they remain in an apical location within the epithelial cell (Fig. 3). Meningococci can be seen in the subepithelial space, but their exact route of access remains unclear. In contrast, when *H. influenzae* is used to infect the NPOC model, the route of mucosal invasion is primarily between epithelial cells (13).

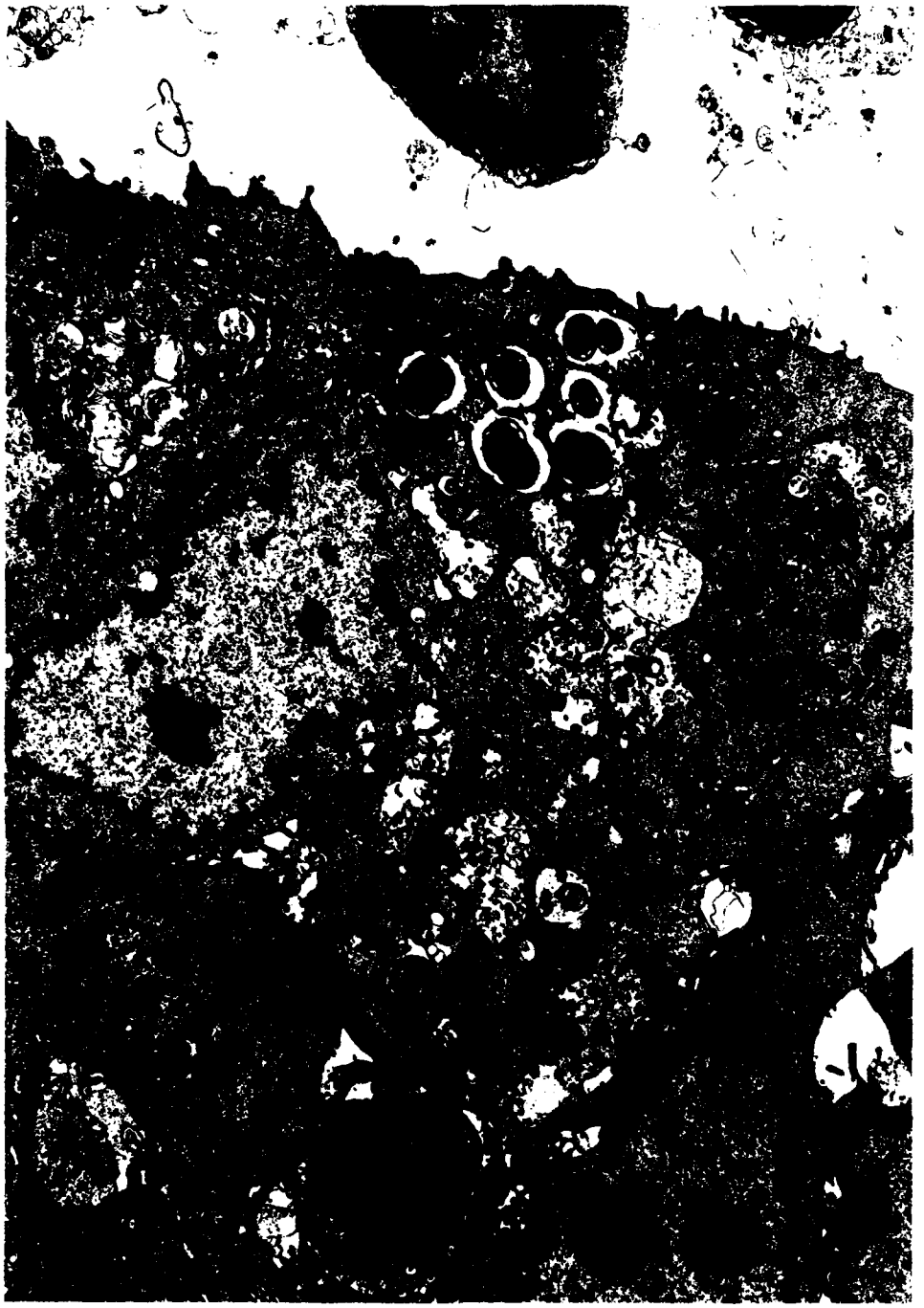

FIG. 3. Transmission electron micrograph showing internalization of meningococci by a nonciliated columnar epithelial cell of human nasopharyngeal organ cultures.

Using the endometrial cell line HEC-1-B, Shaw and Falkow (51) noted that after gonococcal attachment, the organisms were embraced by microvilli and entered these cells in membrane-bound vacuoles. However, 8 h after infection, gonococci were found in the cytoplasm free of membrane-bound vacuoles. At 12 h, hundreds of internalized gonococci were noted free in the cytoplasm. Lysis of the invaded cells was also noted. In contrast, *N. lactamica* was adherent but not invasive in this model and *N. sicca* was not invasive for HeLa cells (2).

Outer membrane blebs are also internalized by epithelial cells (9). LPS antigens on ingested meningococci and gonococci or on blebs may remain unaltered after internalization (J. F. L. Weel, S. Gigengack, C. T. P. Hopman, Y. Pannekoele, and J. P. M. van Putten, personal communication), in contrast to the processing that may occur after ingestion by polymorphonuclear leukocytes.

The molecular events that lead to internalization of gonococci and meningococci are also being actively studied. Engulfment requires viable organisms (46). Engulfment was inhibited when cell microtuble- or microfilament-dependent movement was disrupted by cytochalasin B or demecolcine.

Gonococci and meningococci appear to enter epithelial cells by a process called parasite-directed endocytosis (37). The process involves a mechanism similar to classical phagocytosis (microfilament dependent, blocked by cytochalasin) but appears to be dependent on microbial factors for initiation and occurs with host cells that are not normally phagocytic. Pili are not required for entry. James (24) noted the adherence of gonococci expressing PIIs (opaque colonies) to human embryonic fibroblast cell cultures, with the subsequent formation of microcolonies. Transparent variants (which presumably lacked PIIs), produced at the periphery, were observed to translocate across the tissue cultures by twitching mobility and were more invasive. Other studies have found no differences in the invasiveness of attaching PII^+ or PII^- gonococci (2).

The major porin proteins (gonococcal proteins I [PIA and PIB] and meningococcal class II and III proteins) have been proposed as candidates for the gonococcal and meningococcal invasin. The porin proteins have been shown to insert into lipid bilayers (34). Layh et al. (33) found that gonococcal PIA inserted into mammalian cells identically to its orientation in the gonococcal membrane. Heckels et al. (23) noted that monoclonal antibodies to gonococcal PIA and PIB blocked cytotoxicity and invasion of Chang conjunctival epithelial cells.

Events after Bloodstream Invasion

Few studies with human cells, cell cultures, or organ cultures have addressed events (e.g., endothelial cytotoxicity, penetration of the blood-brain barrier) that occur after bloodstream invasion by gonococci or meningococci. Pathologic specimens suggested that events similar to those noted at mucosal surfaces occurred after dissemination. For example, gonococci have been seen inside A cells of the synovial membrane (14), and gonococcal peptidoglycan monomers have been implicated in cell damage at synovial membranes (42, 47). Wispelwey et al. (B. Wispelwey, A. J. Hesse, E. J. Hansen, and W. H. Scheld, Clin. Res. 35:495A, 1987), using a model of isolated cerebral capillaries from the rat brain, showed that the LPS of *H. influenzae* altered the permeability of the blood-brain barrier. Whether meningococcal, but not gonococcal, LPS produces similar results would be of great interest.

SUMMARY

Human cells, cell cultures, and organ cultures have been extremely useful for studying the events that occur when gonococci and meningococci encounter human mucosal surfaces. The specificity and selectivity of these events for human cells are striking and correlate with the adaptation of these pathogens for survival on human mucous membranes. To colonize these sites, meningococci and gonococci have developed mechanisms to damage local host defenses such as the mucociliary blanket, to attach to epithelial cells, and to invade these cells. Attachment to epithelial cells mediated by pili, and to some types of cells mediated by PIIs, serves to anchor the organism close to sources of nutrition and allows multiplication. Intracellular invasion, possibly initiated by the major porin protein, may provide additional nutritional support and protection from host defenses. Mucosal invasion may also result in access of gonococci and meningococci to the bloodstream, leading to dissemination.

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LITERATURE CITED

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Molecular Basis for Serum Resistance in *Neisseria gonorrhoeae*

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The molecular basis for resistance to killing by normal human serum (NHS) exhibited by certain strains of *Neisseria gonorrhoeae* represents a composite of numerous phenomena. These include (i) the absence in human serum of bactericidal antibody directed against specific epitopes, the most important of which reside in lipooligosaccharide (LOS) antigens (11, 52, 59); and (ii) phenotypic shifting of LOS epitope expression (41, 59), such as may occur especially in vivo (10). This may result in the loss of epitopes that serve as appropriate receptors (bactericidal or lytic epitopes) for bactericidal antibodies and complement. Alternatively, new epitopes may be acquired in vivo, which may obscure underlying lytic epitopes, thereby preventing recognition of these by human bactericidal antibodies. In addition, (iii) blocking antibodies, directed at outer membrane protein antigens, modulate or down-regulate bacterial killing, mainly by preventing binding of bactericidal antibodies to lytic epitopes (26, 49). Finally, and least well understood, (iv) there is a partial failure to form the C5 convertase necessary for full development of membrane attack complexes (MACs or C5b-9 [11, 12]) but also a failure of MACs that do form to fully insert through the gonococcal outer membrane (22, 27). In addition, less C3 may bind to serum-resistant organisms, and C3 that does bind may not be hemolytically active (51).

IN VIVO (UNSTABLE) SERUM RESISTANCE

N. gonorrhoeae strains are phenotypically resistant to killing by NHS in vivo. When subcultured, many strains lose this property and become serum sensitive, hence the term unstable serum resistance. Studies performed by Ward et al. in 1970 demonstrated that *N. gonorrhoeae* harvested directly from a urethral exudate resisted killing by serum obtained from an infected patient as well as by immune sera prepared in rabbits (62). It has not been determined whether human sera with antibody directed against a putative bactericidal or lytic epitope(s) that may be expressed in vivo will overcome unstable serum resistance and kill these organisms. Nevertheless, *N. gonorrhoeae* organisms that maintain the resistant phenotype after in vitro subculture (stable serum resistance) can be killed by appropriate human immune sera, for example, serum samples from patients recovering from disseminated gonococcal infection (DGI) (48, 53).

In vivo serum resistance may be simulated in vitro by incubating serum-sensitive organisms in ultrafiltrates derived from guinea pig sera (36, 50) or human fluids, including sera (33, 34) and genital secretions (cervical secretions and seminal plasmas [35]). After incubation in these fluids, serum-sensitive organisms become serum resistant, having undergone changes in their LOSs. This is evidenced by shifts in LOS migration patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (59), alteration of pyocin sensitivity patterns (63), and reduced binding of normal immunoglobulin M (IgM) to LOSs prepared from strains incubated in these fluids (59).

In vivo serum resistance may result from sialidation of gonococci in the genital tract. Support for this mechanism comes from recent evidence indicating that gonococci grown in vitro in the presence of cytidine 5′-monophospho-*N*-acetylneuraminic acid become serum resistant (42, 44). Gonococci are sialidated at an LOS site (C. A. Nairn, N. J. Parsons, P. V. Patel, J. A. Cole, E. L. Tan, J. R. C. Andrade, M. Goldner, and H. Smith, unpublished observations). Separate evidence that supports in vivo sialidation comes from studies demonstrating that a monoclonal antibody directed against a probable neuraminic acid determinant binds to LOS derived from in vivo- but not in vitro-grown gonococci (9).

IN VITRO (STABLE) SERUM RESISTANCE

Once isolated in culture, *N. gonorrhoeae* undergoes additional phenotypic shifting of antigens, in large part but not exclusively in LOS. At this time, strains assume a range in susceptibility to killing by NHS. This pattern of in vitro susceptibility, however, is relatively stable under constant growth conditions and is an important harbinger of the clinical potential of strains. *N. gonorrhoeae* strains are capable of causing a diverse array of syndromes in the hosts they infect. The syndromes range from the total absence of signs or symptoms at the local site of infection, as occurs commonly in DGI, to a marked local inflammatory response, as exemplified by acute salpingitis. The most solidly established attribute that bears on the inflammatory potential of the organism at the local site of infection is serum resistance (7, 11, 12, 13, 45, 48, 53). This property may confer upon the organism the ability to escape local defenses and enable it to penetrate mucosal barriers to reach the bloodstream and then to disseminate. Organisms with the stable serum-resistant phenotype often persist at local sites of infection without promoting clinically significant inflammation. This is evidenced by a disproportionate representation of these strains in asymptomatic men (7) and by the frequent lack of local symptoms that accompany disseminated infection (43, 45). Differences in stable serum resistance may subdivide DGI strains into two populations; those more resistant may cause arthralgias and tenosynovitis, and those less resistant may cause suppurative arthritis (43). In contrast, *N. gonorrhoeae* strains with the stable serum-sensitive phenotype are often associated with symptomatic local infection in both men and women, and sensitive organisms may be especially associated with the severity of the inflammatory response in women with acute salpingitis (48).

Serum-sensitive gonococci more rapidly generate C5a, the complement-derived neutrophil chemoattractant, when incubated in NHS than serum-resistant organisms do (12). They are also more rapidly and completely phagocytosed by polymorphonuclear leukocytes in vitro than serum-resistant strains are (51). Thus, generation of an inflammatory response by gonococci with the stable serum-sensitive phenotype appears to be conducive to the localization of disease and the prevention of bacteremic spread of infection, as evidenced clinically by the rarity with which acute salpingitis leads to DGI (3, 37, 43).

LOS

The structural and chemical makeup of gonococcal LOS molecules undoubtedly influence stable serum resistance. When gonococci are grown in vitro under glucose-limiting conditions, saccharide structures of gonococcal LOSs are modified. Serum-resistant organisms grown under these conditions in continuous culture remain serum resistant while high dilution rates are maintained. At lower dilution rates, LOS serotype antigen expression decreases and new LOS determinants appear, which may serve as bactericidal or lytic epitopes, and the organisms become more serum sensitive (41). In this case, serum sensitivity may result from newly formed determinants that serve as appropriate receptors for bactericidal antibody. Alternatively, bactericidal or lytic epitopes that previously were inaccessible or cryptic may become uncovered or more surface exposed. In either case, LOS structure undergoes measurable change, indicating that the oligosaccharide chemistry of these molecules is influenced by changes in growth conditions. Oligosaccharide preparations from LOSs of serum-sensitive *N. gonorrhoeae* strains, separated into larger *N*-acetylglucosamine-rich and smaller *N*-acetylglucosamine-poor components, have different effects in complement-dependent bactericidal systems. When used as immune inhibitors in IgM-dependent bactericidal assays, only the larger *N*-acetylglucosamine-containing molecules inhibit serum-bactericidal activity (20). This inhibition is due, in part, to the reduction of IgM LOS antibody by immune absorption, but activation of the alternative pathway of complement also occurs (24). Hexosamines containing LOS molecules are important activators of the alternative pathway (21).

Numerous studies have confirmed that specific antibodies directed against LOS antigens of *N. gonorrhoeae* are responsible for complement-dependent bactericidal activity (17, 46, 60, 61). This activity has been ascribed to LOS antibodies of the IgM class in NHS, directed against serum-sensitive strains (1, 2, 17). In addition, anti-protein I (PI) antibodies contribute to bactericidal activity, which is otherwise primarily influenced by LOS antibodies (23).

Serum-resistant strains also contain bactericidal epitopes. These may not be functional, because NHS lacks antibody to these sites (52) or because these epitopes may be cryptic (Rice et al., unpublished). Nevertheless, serum-resistant *N. gonorrhoeae* strains may be made serum sensitive with the appropriate antibody (32, 46, 53). At least two accessible LOS lytic or bactericidal epitopes are present on serum-resistant gonococci. One of these is defined by monoclonal antibody 2-1-L8, which binds to most strains that resist killing by NHS but to few that are sensitive. This determinant resides on a 3.6-kilodalton (kDa) species of LOS. Seemingly paradoxically, monoclonal antibody 2-1-L8 is bactericidal for serum-resistant strains (epitope present) but not for serum-sensitive strains (epitope absent) (52). Likewise, serum from DGI patients that contains antibody with 2-1-L8 specificity also kills serum-resistant gonococci (Rice et al., unpublished). A second LOS lytic epitope that permits killing of serum-resistant gonococci has been defined by immune serum obtained from an individual administered *Escherichia coli* J5 vaccine. This serum was capable of killing *N. gonorrhoeae* that resisted killing by NHS, but it lacked specificity for the 2-1-L8 epitope (8). In serum samples from both the DGI patients and the J5 vaccinee, bactericidal LOS antibody was IgG and not IgM (8).

Serum-resistant strains may harbor other epitopes that would serve as sites of recognition by bactericidal antibodies present in NHS if the epitopes were exposed. These epitopes may be obscured by LOS structures that prevent accessibility of antibody to these cryptic determinants. An examination of a series of pyocin mutants differing by sequential saccharide deletions in LOS (15) has revealed that as the saccharides shorten, exposure of the 2-1-L8 epitope increases. The organisms remain serum resistant as long as the 2-1-L8 epitope is present. As the 2-1-L8 epitope abruptly disappears from one mutant to another with a shorter LOS, the organisms convert to being serum sensitive as underlying serum-sensitive LOS epitopes become exposed. IgM binding to LOS also increases markedly as the 2-1-L8 epitope is lost and the cryptic epitopes emerge as exposed determinants (Rice et al., unpublished). These results have been corroborated by using the pyocin mutant LOS molecules themselves, isolated from whole organisms. Mutant LOSs of various molecular masses have been inserted into liposomes and used as antigenic targets. As the mass of LOS used in the liposomes increases beyond 3.6 kDa (where the 2-1-L8 epitope resides), the liposomes become more susceptible to antibody-mediated MAC insertion, despite the presence of the underlying 2-1-L8 epitope. If the liposome assay is used as an analog to serum killing of gonococci (Rice et al., unpublished), these results suggest that the presence of the 2-1-L8 epitope may not always preclude serum sensitivity. Indeed, certain pyocin mutants with longer oligosaccharide chains but also harboring the 2-1-L8 epitope may sometimes be serum sensitive (P. A. Rice and M. A. Apicella, unpublished observations). A genetic approach, i.e., selection of transformants on the basis of their strong reactivity with monoclonal antibody 2-1-L8, produces serum-resistant clones. However, the level of resistance is defined by the loss of higher-molecular-mass LOS components (56). Other reports have also indicated that changes in the molecular mass of LOS may alter serum resistance (40, 55, 57, 59). The loci on the gonococcal chromosome designated as *sac-1* (5, 6) and *sac-3* (54) are associated with serum resistance, and *sac-3* in particular has been shown to affect the molecular mass of LOS (57). Recently, a recombinant plasmid, called pWM3, was created from a cosmid gene library of *N. gonorrhoeae* and used to confer serum resistance upon transformation of a serum-sensitive strain (39). The deoxyribonucleic acid sequence of this cloned region has now been determined, and hybridization probes have shown the homologous gene sequences to be present in several strains of *N. gonorrhoeae*, including some that are phenotypically serum-sensitive (C. J. Conde-Glez, W. M. McShaw, S. Nowicki, S. I. Hull, and R. A. Hull, unpublished observations). These regions are probably separate from *sac-1* and *sac-3* loci.

PIII and Blocking Antibody

Natural antibodies of the IgG class may subvert adequate insertion of the MACs (or C5b-9) of complement and therefore contribute to serum resistance. These antibodies have been described for human sera and are termed blocking antibodies (26, 38, 47, 49). They interfere with the efficient insertion of the MAC by an as yet unknown mechanism(s); however, binding of these antibodies to particular antigenic targets on the surface of gonococci may divert the necessary localization of complement away from bactericidal sites (26). Recent studies with murine monoclonal antibodies have shown that an antibody specific for a gonococcal surface protein, protein III (PIII), is able to block killing of gonococci by bactericidal antibody directed against a separate

epitope (29). Human antibodies that are specific for PIII also block killing of *N. gonorrhoeae* by bactericidal antibody. Immunodepletion of PIII antibody from certain DGI sera that lack killing ability may restore their killing action for serum-resistant gonococci (49). In some instances the depletion of PIII antibody from NHS enables absorbed serum to kill gonococci that are otherwise serum resistant (P. A. Rice, S. Gulati, E. C. Gotschlich, L. M. Wetzler, and M. S. Blake, unpublished observations). This suggests that NHS may contain antibody that is bactericidal for all strains of *N. gonorrhoeae* but that this antibody may not always function. Through the use of genetically altered *N. gonorrhoeae*, a PIII deletional mutant (PIII$^-$) has been shown to be more serum sensitive than its parent. The relative difference in serum sensitivity between parent and mutant is directly related to the quantity of PIII antibody present in the sera used in the bactericidal assay (P. A. Rice, S. Gulati, E. C. Gotschlich, L. M. Wetzler, and M. S. Blake, unpublished). PIII not only appears to be present in all strains of gonococci (30, 31) but also appears to be biochemically and immunochemically identical among strains (31, 58). It is also a potent immunogen in humans. Contaminant amounts of PIII present in a PI vaccine preparation were capable of eliciting PIII blocking antibodies when administered to humans (F. Arminjon, M. Cadoz, S. A. Morse, J. D. Rock, and S. K. Sarafian, personal communication).

PIII has extensive homology to the enterobacterial OmpA protein (18) and class 4 meningococcal protein (K. P. Klugman, and E. C. Gotschlich, unpublished observations). Since members of the family *Enterobacteriaceae* and *N. meningitidis* are both common human commensal organisms, it is possible that PIII blocking antibodies arise as a result of exposure to cross-reacting proteins from other species. Human IgG directed at OmpA not only binds to gonococcal PIII but also promotes blocking activity (P. A. Rice, S. Gulati, E. C. Gotschlich, L. M. Wetzler, and M. S. Blake, unpublished). Hence, blocking antibodies may arise naturally from exposure to members of the family *Enterobacteriaceae* and perhaps to *N. meningitidis*.

Transformation of serum-sensitive *N. gonorrhoeae* strains with the plasmid pWM3 (see above) or subclones that encode only a 29-kDa protein enable the derivatives to bind blocking antibody. Although similar in size, the cloned 29-kDa protein and PIII are antigenically distinct, and sequences of deoxyribonucleic acid encoding PIII and the 29-kDa protein differ (Conde-Glez et al., unpublished). pWM3 also encodes a 17.5-kDa protein, but its role is presently unknown. The 17.5-kDa protein is not a binding site for blocking antibody (39).

Activation and Disposition of Complement

MACs (or C5b-9) form on the surfaces of both serum-sensitive and serum-resistant organisms (22, 27). Both sensitive and resistant strains consume equivalent amounts of C9 and bind similar numbers of C7 and C9 molecules when incubated in NHS (22, 27). However, two features distinguish the binding of C5b-9 to serum-sensitive versus serum-resistant strains (28). First, C5b-9 is more sensitive to trypsin on serum-resistant strains (>twofold), indicating that MACs are not inserted identically in serum-sensitive and serum-resistant organisms. There may be differences in the extent of insertion of C5b-9 complexes, variability in the capacity of trypsin-cleaved C5b-9 to remain surface bound, or differences in the extent of C9 incorporation into the C5b-9 complex. Second, the C5b-9 complex is bound in a different form on serum-sensitive than serum-resistant organisms. The major bactericidal form of C5b-9 on the surface of serum-sensitive *N. gonorrhoeae* strains sediments as a 33S complex when extracted with Zwittergent detergents, but nonbactericidal C5b-9 complexes on serum-resistant *N. gonorrhoeae* strains exist as larger aggregates of C5b-9 or as C5b-9 complexed to bacterial outer membranes. Presensitization of serum-resistant *N. gonorrhoeae* with immune rabbit serum that is bactericidal converts serum-resistant organisms to serum-sensitive ones, alters the release of C5b-9 by trypsin, and changes the sedimentation properties of C5b-9 complexes to coincide with those seen in serum-sensitive organisms incubated in NHS (27). Bactericidal and nonbactericidal complexes (C5b-9) bind to identical radiolabeled protein components of the outer membrane; however, bactericidal C5b-9 also binds to additional proteins (28). Taken together, these studies have suggested quantitatively but not qualitatively the equal presence of C5b on the surfaces of serum-sensitive and serum-resistant gonococci. Binding to other constituents of the outer membrane, particularly LOSs, has not been reported.

Separate studies have examined the generation of C5a; the formation of C5a is stoichiometrically related to C5b formation. These results suggest a different interpretation of the data from that indicated above. When phenotypically stable serum-resistant strains are incubated in NHS, C5a generation is significantly lower than when serum-sensitive strains are used (11), yet C5b binding is the same for both phenotypes (22, 27). Nevertheless, immune serum from DGI patients, particularly IgG LOS antibody that converts organisms from the serum-resistant phenotype to the serum-sensitive phenotype, permits additional generation of C5a by strains that otherwise minimally generate C5a and resist killing by NHS (11). Perhaps the discrepancy between unequal C5a generation by each of the two phenotypes versus equal C5b binding may be explained by differences in the C5 convertases present in each case. This difference may produce altered efficiency of C5b adherence that results in diminished C5b binding to serum-sensitive organisms, despite a given amount of C5a release. Effective MAC formation may also play a role in C5a generation. Perhaps incubation of serum-sensitive organisms in C8-deficient serum, which cannot kill gonococci, would result in decreased release of C5a compared with that obtained after incubation in NHS. Such is the case in analogous systems in which sensitized sheep erythrocytes are used instead of bacteria. Replacement of C8 in these systems restores the levels of released C5a (19).

The effect of MACs on earlier stages of the complement system is also reflected in the kinetics of factor B binding onto serum-sensitive organisms incubated in NHS versus C8-deficient human serum. Although both C3 and factor B binding to serum-sensitive gonococci start out equally in these sera, prolonged incubation (>5 min) results in a decay in factor B binding to serum-sensitive gonococci in NHS but not in C8-deficient serum, in which no effective C5b-9 is formed. C3 binding increases over time and is equal in both sera (14). Selective loss of factor B from gonococcal surfaces incubated in NHS compared with C8-deficient serum could reflect a role of the MAC in promoting the dissociation of the C3bBb-properidin complex or facilitating the action of factor H or I in the dissociating process that results in loss of factor B but maintenance of C3b. This suggests that events that transpire to create effective complement fixation may occur at the point of or before C3 fixation. The presence and quantity of bactericidal antibody are also important in this

case, because C3b can bind to antibody directly, and the function of C3b is thereby enhanced as a result of relative protection from cleavage by factors H and I (4, 16, 25).

The role of gonococcal activation of specific complement pathways has also been investigated. Gonococcal killing by NHS and human serum deficient in properidin proceeds with identical kinetics in both cases (13). Killing in human serum deficient in C2 occurs following a longer incubation period, but subsequently the rate of killing is similar to that observed in NHS and in properidin-deficient serum. These similar killing kinetics may reflect efficient activation of the classical pathway in both NHS and properidin-deficient serum. However, greater C3 fixation occurs in NHS and is due to recruitment of the alternative pathway (13). Gonococci correspondingly increase their ability to bind properidin as they become more serum sensitive (24). The addition of the alternative pathway increases by 40% the effective insertion of MACs by the classical pathway when LOS antigens prepared from serum-sensitive gonococci are inserted into liposomal membranes and used as targets for antibody-mediated insertion of MACs. The alternative pathway alone, however, achieves only 12% of the total insertion of MACs, indicating that the classical pathway facilitates activation of the alternative pathway in this system (M. L. Schulz, P. A. Dale, K. Y. Lung, and P. A. Rice, unpublished observations).

These findings together suggest that C3 may be deposited at different sites on serum-sensitive gonococci, particularly their LOS molecules. This may depend on the complement pathway that is active in the serum. Alternatively, C3 may be deposited at the same site by both pathways, but the efficiency with which it serves as a permissive site for additional C5 convertase formation may differ.

CONCLUSIONS

Resistance of *N. gonorrhoeae* to killing by NHS is probably exhibited by all strains in vivo. After in vitro subculture, however, some strains become serum sensitive, while others remain resistant (stable serum resistance). The molecular basis for resistance to NHS is multifactorial. In vivo resistance may result from sialidation, in vivo, of gonococcal LOSs, thereby providing the organism with a eucaryotic antigenic look against which there may be no human antibody. Strains subcultured in vitro with commonly used bacteriologic media are no longer sialidated but, nevertheless, may remain serum resistant (stable serum resistance). Stable (in vitro) resistance may also be due, in part, to the absence of antibody in NHS directed against surface-exposed LOS bactericidal or lytic sites. However, LOS sites on stable serum-resistant organisms may be suitable for recognition by antibodies present in immune gonococcal sera, thereby rendering these strains sensitive to immune sera. Serum-sensitive strains harbor exposed LOS lytic epitopes recognized from the outset by NHS after organisms are subcultured.

Other mechanisms also contribute to serum resistance. Blocking antibodies directed against outer membrane proteins, such as PIII, prevent binding by bactericidal antibodies and render *N. gonorrhoeae* serum resistant. Serum resistance does not prevent formation of MACs of complement, but, rather, MACs do not fully insert through the outer membranes of serum-resistant gonococci, and death of the organism does not ensue. In addition, the development of C5 convertases is reduced when serum-resistant organisms are incubated in NHS, and this may also contribute to serum resistance.

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Global Epidemiology of Meningococcal Disease

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Meningococcal disease is a significant cause of mortality and morbidity throughout the world (40, 49). Although rates of endemic meningococcal disease range from 1 to 3/100,000 in the United States (26) to 10 to 25/100,000 in many parts of the developing world (47), this disease is noteworthy for causing major, periodic epidemics with attack rates exceeding 500/100,000 (10). The descriptive epidemiology and the patterns of illness of each of the major meningococcal serogroups have been characterized in several recent reviews (26, 40). Current epidemiologic efforts are focused on improving (i) the surveillance for meningococcal disease by using new techniques to identify clonal populations (7, 39) and (ii) the understanding of individual risk factors for illness and antecedents of epidemic disease by using both classical epidemiologic techniques and immunologic methods (24). In this report, we review recent developments in both areas of investigation, emphasizing the continued importance of surveillance (including serogrouping) and a multidisciplinary approach to the analysis of risk factors.

SURVEILLANCE: CHANGES IN THE PATTERNS OF DISEASE

Nine meningococcus serogroups can cause invasive disease, with most illness caused by serogroups A, B, and C. Attack rates are highest in infants from 3 months to 1 year old and then decrease with age. Currently available vaccines are effective in protecting against disease caused by serogroups A, C, Y, and W-135. Infants respond poorly to polysaccharide antigens, however, and vaccination has limited efficacy in preventing disease among those at highest risk. During the past decade, changes have occurred in the patterns of disease caused by each of these serogroups as new strains have emerged and spread.

Epidemic group A meningococcal disease has been documented in various parts of the developing world, with outbreaks beginning during the dry season and ending with the onset of the rainy season. Attack rates generally range from 100 to 500/100,000. Periodic outbreaks occur across sub-Saharan Africa at intervals of 8 to 12 years, with recent outbreaks occurring in Chad and Sudan (in 1988).

Outbreaks in developed countries have been infrequent since a pandemic swept Europe and North America following the Second World War. When group A disease occurs in a developed country, cases are concentrated in the poorest sectors of society (9, 45), reflecting other potential risk factors such as sanitation, crowding, and family size. The most recent such outbreak occurred in Auckland, New Zealand, during the winters of 1985 to 1987. Over 280 cases were reported, with attack rates almost 20-fold higher in the Maori and Pacific Islander communities than among the more affluent New Zealanders of European descent (D. Lennon, L. Voss, D. Hood, and B. Gellin, Pediatr. Res. 23:374A, 1988). Isoenzyme typing, a technique that identifies strains on the basis of the electrophoretic mobility of a panel of enzymes, suggests that the strain responsible for the New Zealand outbreak was the same strain that caused an earlier outbreak in the northwestern United States among skid road inhabitants (B. Gellin, personal communication). Of 14 New Zealand group A isolates from patients with disease between 1980 and 1985, 13 were of the outbreak strain, indicating that this strain had been present in the population for several years prior to the outbreak. The factors that precipitated the 1985 outbreak are not known.

With increased international travel, global dissemination of an outbreak-associated strain may become more common. One example of this potential is provided by an epidemic of group A meningococcal disease occurring in association with the annual Moslem pilgrimage (Haj) to Mecca. Each year, over a million Moslems from throughout the world perform the Haj. In summer 1987, group A meningococcal disease brought into Saudi Arabia by arriving pilgrims spread throughout this gathering, resulting in several thousand cases of invasive disease (46). As the Hajis returned to their home countries, the virulent group A strain was carried throughout the world. Secondary outbreaks among Hajis, their contacts, and, eventually, individuals having no direct contact with Hajis occurred in Saudi Arabia, in other Gulf states (37), and in Pakistan. Although isolated secondary cases occurred in developed countries (11, 43), illness did not spread to the general community. Despite steps taken to prevent future outbreaks during the pilgrimage through vaccination of Hajis, transmission of pharyngeal carriage will not be affected. Other large-scale population movements (e.g., refugees) may present similar problems.

Group B meningococci are recognized to be the major cause of sporadic meningococcal disease in developed countries (26, 40). When outbreaks do occur, the attack rates usually range from 10 to 50/100,000, an order of magnitude less than attack rates during group A outbreaks. During the late 1970s, a group B clone (serotype 15, ET-5 complex) emerged in northwestern Europe and was responsible for outbreaks in Norway, Iceland, Denmark, the Netherlands, and Great Britain (42). Intercontinental spread of this clone had been documented, with outbreaks occurring in Cuba (in 1980), in Chile (in 1985), and currently in Rio de Janeiro and São Paulo, Brazil (7). Strains of this type have also been isolated from patients in the United States, although as of 1988, they have caused only a small fraction of sporadic meningococcal infections.

Group C meningococci have been implicated in large outbreaks (4), small disease clusters (30), and sporadic infections. Changes in the predominant strains and the disease pattern for this serogroup have also occurred. During the 1980s, the proportion of sporadic disease caused by group C organisms increased in several European countries; a single strain was responsible for much of the increase (29). In the United States, active surveillance for meningococcal infections conducted by the Centers for Disease Control in six regions of the United States during 1986 and 1987 found

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group C to be the major serogroup responsible for sporadic disease in two of the regions studied (Los Angeles County and the state of Tennessee). The absolute attack rate of disease in Los Angeles increased threefold as a result of increased rates of group C meningococcal disease. Isoenzyme typing of surveillance isolates from Los Angeles County identified a single clone as being responsible for this increase. Only 1 of 33 strains isolated from patients in 1980 to 1985 was this type. Isolates from Tennessee were divided between the Los Angeles type and a genetically related strain, differing at only one enzyme locus (B. Gellin, unpublished observations).

These changes in strain types and patterns of disease for each of the three major serogroups emphasize the need for continued surveillance and typing of strains isolated during outbreaks and sporadic disease episodes. Serotyping is especially important for group B strains, in which immunity may be type specific rather than group specific, and information about the strains predominating in a population will be important in developing an effective vaccine.

RISK FACTORS FOR MENINGOCOCCAL DISEASE

The second major focus of meningococcal disease epidemiology has been to determine risk factors for meningococcal disease. Although the descriptive epidemiology of endemic and epidemic meningococcal disease has been well documented, individual risk factors for illness are poorly understood. With the availability of preventive measures such as vaccination, predicting who will develop meningococcal disease and determining which risk factors lead to epidemic spread of illness become crucial for targeting intervention to populations at greatest risk.

Progress in characterizing risk factors for meningococcal disease has been slow, because it has been hampered by the infrequent occurrence of outbreaks in developed countries and the difficulty in conducting epidemiologic investigations in developing countries. Moreover, risk factors may vary between developed and developing countries, between endemic and epidemic disease, and among illnesses caused by various serogroups. When studies have been done, results often conflict. Methodologic problems have contributed to the confusion. Misclassification of cases can occur by including individuals without culture-documented meningococcal infection or by including patients with meningitis caused by meningococcal strains not of the epidemic type. Measurement of important variables such as crowding, socioeconomic status, and ventilation are not standardized and differ in various reports. Studies reporting a positive association may have reached a level of statistical significance by chance, whereas those finding no association may have had an insufficiently large sample size to detect a true risk factor.

An additional difficulty in determining risk factors for infection has been that potential risk factors are usually evaluated only as they relate to the occurrence of invasive disease. Before invasive disease develops, however, several steps must occur, beginning with exposure to a carrier, transmission of infection, and establishment of carriage or disease. Risk factors acting at the earlier stages may have only an indirect effect on disease occurrence or, depending on other associated factors, may have no effect at all.

By breaking down the development of meningococcal disease into its constituent steps and evaluating the contribution of potential risk factors at each step, a clearer picture of their impact emerges. We have developed a model

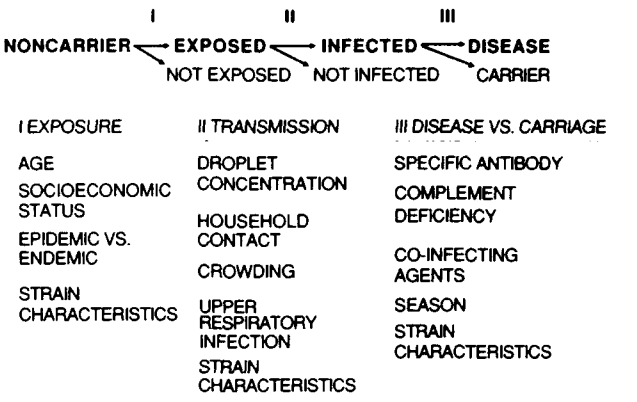

FIG. 1. Factors determining the occurrence of meningococcal disease.

for meningococcal infection that examines the sequence of events ending with invasive disease and have evaluated the role of potential risk factors at each step in the sequence. Although this model provides a framework for the evaluation of various potential risk factors, it also makes clear the gaps in our understanding of meningococcal disease epidemiology. The model includes three stages: first, exposure to a meningococcal carrier; second, acquisition of infection; and, third, development of carriage or invasive disease (Fig. 1).

Stage I: Exposure

Humans are the only natural host for meningococci. Therefore, infection can be acquired only after exposure to a carrier of the organism. The likelihood of contact with a carrier depends, in part, on the prevalence of carriage in the population. Reported carriage rates vary widely among studies, which have been conducted in both developed and developing countries during periods of both endemic and epidemic disease.

During periods of endemic disease in the United States, Greenfield et al. found an overall carriage rate of 5.7% (20), whereas Gold et al. found rates between 0.6 and 2.0% (17). In both studies, the most common groupable strains were serogroup B followed by serogroup Y. No group A strains were isolated in either study. Meningococcal carriage rates were highest in older children and young adults. In contrast, *N. lactamica* was most commonly found in children between 1 and 4 years old.

Three European studies evaluated carriage during outbreaks of serogroup B meningococcal disease. A survey conducted in two Belgium schools during a serotype B2 outbreak showed a carriage rate of 9.8% in a school with a student population that was of moderate to high socioeconomic class and a carriage rate of 32.6% in a school serving families of lower socioeconomic class. Most isolates from both schools were group B or W-135 (12). Serotyping of carriage strains and comparison with the outbreak strain were not done. During an outbreak of serotype B15 disease in England, a community survey showed 10.9% of individuals to be meningococcal carriers, with 1.4% carrying the outbreak strain. Among schoolchildren, rates were 12.6 and 1.5%, respectively (6). Similar rates were obtained by Caugant et al. during an outbreak in Norway (8). They found an overall carriage rate of 10.1%, with only 0.7 and 0.9% being carriers of the strains causing over 90% of the invasive disease.

In developing countries, carriage rates also vary with age, socioeconomic status, the presence of an ongoing epidemic, and the particular strains that are predominant in the area. During a nonepidemic period in northern Nigeria, 10% of those surveyed were carriers, with the highest rates occurring in young adults. Strains most frequently isolated were group B (58%) and group C (24%); only 11% were group A. No seasonal variation in carriage rate was noted (36). Blakebrough et al. examined carriage at a school and its surrounding village in northern Nigeria at times of both endemic and epidemic group A disease (2). Before the outbreak, 7% of schoolchildren were carriers, primarily of group C strains. During the outbreak, the overall carriage rate was not significantly different (9%), but a larger proportion of isolates were group A (72% versus 13% before the outbreak); 9% of children also carried *N. lactamica*. The village survey showed similar results, with a shift to serogroup A carriage during the outbreak period. Carriage studies during a previous group A outbreak in Nigeria gave similar results, identifying 7.4% of the population as meningococcal carriers, of whom 35% carried group A strains (27).

Strain characteristics also influence carriage rates. Clonal analyses of group B strains show different rates of carriage and disease for different strains. The duration of carriage varies with serogroup as well. Studies in developed and developing countries during periods of endemic disease show low rates of group A carriage relative to the other serogroups. However, since culture surveys measure the point prevalence of carriage, the rate is dependent on both the acquisition rate and the duration of carriage. Studies from Saudi Arabia and Nigeria document a mean duration of 1 month for group A carriage (2, 9). The Nigerian study showed a significantly longer duration for carriage of nongroup A *N. meningitidis*. Although the prevalence of group A carriage may be low, the incidence (the number who acquire this organism during a given period) tends to be relatively high. The high incidence of occult infection, as measured by antibody studies (2), increases the likelihood of exposure to someone carrying a strain from this serogroup. Reasons for the difference between serogroups in duration of carriage are not known.

Results of these carriage surveys suggest several conclusions: (i) carriage rates are highest in school-age children and young adults; (ii) carriage rates may be higher in persons of low socioeconomic status; (iii) carriage rates do not vary with the seasons; (iv) strains isolated during interepidemic and epidemic periods are heterogeneous, with a shift to group A carriage during group A outbreaks (this shift to carriage of the epidemic strain did not occur during group B outbreaks that have been studied); and (v) strain characteristics affect carriage rates, with carriage strains not necessarily corresponding to strains causing invasive disease.

Stage II: Acquisition of Infection

Not all individuals exposed to a meningococcal carrier will acquire infection. Meningococci are spread via respiratory droplets. Therefore, transmission of infection requires aspiration of infective particles by noncarriers. Factors that increase the likelihood of this include those that increase the number of aerosolized particles, prolong survival of the meningococci in the droplet, and increase the chance of contact of noncarriers with infective particles. The immunologic status of potential hosts may also be important, a factor that will be reviewed briefly. Organism-related factors, especially those affecting attachment to the pharyngeal mucosa, are also likely to be important.

Formation of respiratory droplets is enhanced by coughing and sneezing. If carriers express symptoms, either related to an intercurrent viral upper respiratory infection or produced by the carriage itself, the formation of respiratory droplets would be increased. Olcen et al. obtained pharyngeal cultures from 64 family members of patients with meningococcal disease, 25 (39%) of whom were carriers, with 22 carrying the same strain as the patient (38). Of 24 carriers interviewed, 20 (83%) reported upper respiratory infection symptoms, compared with 13 (35%) of 37 noncarriers. Similar results were noted by Moore et al., who found fever, sore throat, and cough to be more common in carriers of group A meningococci than in noncarriers (34).

Following the aerosolization of particles containing meningococci, factors that increase the survival of the organism in the environment may increase the likelihood of transmission of infection. A field study to evaluate the effect of season and ventilation on aerosolized bacteria was conducted in Mali and Burkina Faso in 1968 to 1969, in which air samples were obtained from various types of dwellings during both the dry and rainy seasons. Although no meningococci were isolated, greater concentrations of viable bacteria were present during the dry season (humidity, 18 to 68%) than during the rainy season (humidity, 56 to 92%) (16). The temperatures were similar in both seasons. No conclusions could be reached regarding the effect of ventilation on the indoor concentration of airborne bacteria. We know of no studies of the effect of temperature on the survival of meningococci in the environment, but expect that desiccation may occur more rapidly at higher temperatures.

Acquisition of infection depends not only on the concentration of infective particles in the environment but also on the chance that a noncarrier will inhale those particles. Household contact with a carrier was shown to increase the acquisition rate from 0.7% per month to 1.6% per month during an epidemic in northern Nigeria (2). A study conducted during a group C epidemic in Brazil showed a higher carriage rate in persons living with a patient who had meningococcal disease than among those who visited or worked in the household (35). Hassan-King et al. found not only a higher carriage rate in family members than in other household contacts, but also a higher carriage rate among persons who slept in the same room as a patient with meningococcal disease (27). Transmission also increases with increased crowding within a household. During the 1987 group A meningococcal disease outbreak in Saudi Arabia, 33% of persons in households having more than two persons per room were carriers, compared with only 20% of persons in households having less than two persons per room. Bedroom crowding was also associated with increased rates of carriage (B. Schwartz, unpublished observation).

The role of immunologic factors in establishing an infection is not clear. The presence of group-specific antibody elicited by vaccination does not affect the carriage rate (1, 3). Simultaneous carriage of more than one strain can also occur (3).

Stage III: Carriage versus Invasive Disease

Risk factors for invasive disease in persons with meningococcal infection are not completely understood. A combination of host factors, environmental factors, and organism characteristics may be important in affecting the balance between carriage and disease.

Invasive meningococcal disease occurs primarily in persons who are newly infected with the organism. In studying military personnel, Edwards et al. found that 31 (86%) of 36 patients had negative nasopharyngeal cultures during the 2 weeks before becoming ill and that 4 of these were culture negative the day before developing disease (14). The remaining 5 of 36 patients had positive cultures less than 4 days prior to the onset of illness. Moreover, meningococcal outbreaks occur not at times of high pharyngeal carriage but when the rate of acquisition of infection is increased (48).

The presence of serum bactericidal antibody (immunoglobulins G and M) is probably the most important host factor preventing invasive disease. In a seminal series of studies, Goldschneider et al. demonstrated a clear correlation between bactericidal antibody titers and host immunity (18). They found that in a large cohort of Army recruits, 94% of soldiers who subsequently developed meningococcal disease had group-specific bactericidal titers below 1:4, in contrast to healthy controls. Interestingly, meningococcal patients also had significantly lower group-specific bactericidal titers against heterologous serogroups, indicating that individual differences in generating an effective immune response may play an important role in disease susceptibility. The high rates of meningococcal disease in military recruits during the prevaccination era presumably resulted from bringing new recruits together, many of whom did not have previous exposure to the invasive serogroup or were not able to mount an effective humoral response. Disease rates in veteran soldiers, however, were much lower than in new recruits (5). The role of natural immunity in prevention of invasive disease also explains the high attack rates seen in younger age groups. Peak attack rates occur in infants of 6 to 9 months old, an age when maternally acquired antibodies are being lost. Carriage of *N. lactamica* and other nonpathogenic *Neisseria* species may provide immunity by stimulating protective antibodies that cross-react with pathogenic strains (17, 19).

Although specific antibody is generally protective, this immunity is not absolute. During an epidemic in the Gambia, Greenwood et al. documented illness in individuals with preexisting antibody titers considered protective (22). Overall, 4 of 25 patients had high bactericidal titers before becoming ill. Kayhty et al. measured group-specific antibody titers and the proportion of antibodies in the different immunoglobulin classes in acute-phase serum samples from Finnish patients with group A or C meningococcal disease at the time of hospital admission (31). They found that 16% had antibody levels deemed protective.

The occurrence of disease in persons with preexisting "protective" antibody levels has been addressed by Griffiss, who hypothesized that the activity of bactericidal antibodies might be blocked by immunoglobulin A antibodies induced either by other meningococcal strains or by cross-reacting enteric or respiratory bacteria (23). This mechanism postulates that since immunoglobulin A does not bind complement, it may block binding sites for other bactericidal antibody classes. Accordingly, outbreaks of disease might reflect transmission of cross-reacting organisms in previously immune populations.

Immune lysis by complement also plays an important role in protection from meningococcal disease. Therefore, persons with complement deficiency may develop disease despite protective antibody. Ellison et al. found primary or secondary deficiencies in 6 (30%) of 20 individuals with sporadic infection in the United States (15). Studies conducted during group A epidemics in Africa, however, indicated that this was not a significant factor in determining who developed disease (22).

The condition of the host pharyngeal mucosa and respiratory epithelium may also be important in protection from invasive disease. Concurrent viral upper respiratory infections may denude the mucosa (32) and increase invasion by the organism. Sporadic cases and outbreaks of meningococcal disease have clearly been associated with concurrent viral upper respiratory tract illness (33, 50). During an epidemic in Chad, patients with meningococcal disease were found to shed respiratory viruses and mycoplasma at a significantly higher rate than age- and sex-matched controls (P. Moore, unpublished observation). An investigation of a simultaneous outbreak of meningococcal disease and influenza showed that both meningococcal carriage and disease were significantly more common among patients with serologic evidence of influenza infection, despite similar levels of exposure between patients with and without the viral illness (50).

Other factors may also affect the integrity of the respiratory mucosa, degrading its effectiveness as a barrier to invasion. One explanation for the seasonality of epidemic group A disease is that during the dry season the mucosa is chronically irritated. This problem may be exacerbated by periodic dust storms. During the 1988 outbreak in Chad, the rate of disease had been declining until a dust storm occurred that was followed by a subsequent increase in cases (T. Lippeveld, personal communication), and Greenwood et al. found a significant association between the number of cases of meningococcal disease in Nigeria and the intensity of the harmattan, a dry, dusty wind from the Sahara (21). Exposure to cigarette smoke was also found to be related to developing disease (25).

Strain characteristics also affect the balance between carriage and disease (28). Different serogroups are clearly responsible for different patterns of meningococcal disease. Within serogroups, some strains are more closely associated with epidemic disease, whereas others are less likely to cause infection. Olyhoek et al. used isoenzyme electrophoresis and monoclonal typing to distinguish clonal populations in a large series of group A meningococcal isolates from 28 different epidemics (39). In all but one epidemic, a single clonal population was responsible for illness. Several clones were implicated in multiple epidemics, whereas others were unrelated to epidemic disease. Differences in the disease-to-carriage ratio have been shown for group B strains as well (8).

The effect of environmental factors on the risk of invasive disease is difficult to evaluate, since the same factors may affect the risk of exposure to a carrier and the likelihood that transmission will occur. For example, household contact with a carrier increases the transmission of infection (stage II) and also increases the occurrence of disease. Although carriage rates in household members of patients are increased approximately threefold (46), rates of disease in household members are increased several hundredfold (13). Household crowding may independently affect both the transmission of infection and the occurrence of disease (44). Transmission of potential cofactors, such as cross-reacting organisms or upper respiratory infection agents, could be increased in more crowded conditions. Since several different meningococcal strains are present in the population at any time, the occurrence of secondary cases in a household may be only a marker for the presence of a strain more likely to cause disease in that family. It is also possible that

increased household attack rates reflect a common genetic susceptibility to disease in family members.

OCCURRENCE OF MENINGOCOCCAL EPIDEMICS

Epidemics of meningococcal disease are composed of individual cases clustered in time and space. Therefore, factors that precipitate an epidemic must also be risk factors for individual cases. Since carriage rates do not correspond to the rate of invasive disease and may not increase significantly during an epidemic, risk factors for epidemic disease are likely to be those affecting the balance between carriage and invasive disease (stage III in the model).

A combination of factors must exist for an epidemic to occur. The descriptive epidemiology of meningococcal outbreaks provides a clue to what factors might be important. For example, periodic outbreaks of group A meningococcal disease occurred in both the United States and Africa before the 1950s. Although major epidemics in sub-Saharan Africa continue to follow this pattern, outbreaks in developed nations are infrequent and are generally restricted to the poorest sectors of society. Meningococcal outbreaks also tend to be seasonal, and the mean age for patients during an outbreak is above that for those with sporadic disease (41). These features suggest that socioeconomic status (or a correlate, such as sanitation or crowding), season, and immunity are important factors in the occurrence of epidemic disease. Strain characteristics are important as well. The introduction of a virulent strain into a previously unexposed population, as may have occurred during the Haj, may also precipitate a meningococcal outbreak. The clonal strain causing this outbreak, designated A-1 by Olyhoek et al. (39), was probably imported into Mecca by Asian pilgrims and subsequently caused a major epidemic in Chad and Sudan in 1988 (Moore, unpublished). However, the presence of a virulent strain alone is usually not sufficient to result in an epidemic.

Risk factors for epidemic disease can be divided into two groups: factors that are permissive (i.e., necessary but not sufficient for an outbreak to occur), and factors that act to initiate an outbreak. Immunologic susceptibility, appropriate climatic conditions, low socioeconomic status, and transmission of a virulent strain appear to be necessary for an outbreak to occur. If these conditions are present, an outbreak can then be precipitated by an initiating factor, such as exceptional climatic conditions (excessively dry season, dust storms) or the spread of an infectious cofactor. In the latter case, the spread of a respiratory pathogen, not meningococci, might be the primary initiating factor for a meningococcal epidemic.

No longitudinal data exist to document changes in group- or type-specific specific immunity over time. Because of the importance of immune status in the development of invasive disease, however, waning herd immunity to a particular strain in a population seems necessary for an outbreak to occur. Although an outbreak of a coinfecting organism that induces cross-reacting antibodies might cause a meningococcal outbreak in an immune population, studies during outbreaks in The Gambia (22) and in Finland (31) indicate that only a small proportion of cases occur in persons with protective antibody titers.

The role of climatic factors in precipitating outbreaks is unclear. Although outbreaks occur during the dry season, many dry seasons can pass without epidemic disease. Some evidence suggests that the year preceding an epidemic may be drier than average. Evaluation of historical rainfall data from Burkina Faso and Mali since the 1940s found this pattern for four of six outbreaks (D. Le Comte, unpublished observations). Additional data must be evaluated to determine whether this pattern is consistent for other group A outbreaks.

Socioeconomic factors, being constant over time, are probably not directly related to the occurrence of outbreaks. An outbreak of meningococcal disease could be precipitated by an outbreak of a concurrent infection, however, which is more likely among those living in poorer, more crowded conditions. This hypothesis also needs further study.

CONCLUSIONS

The role of potential risk factors for meningococcal disease can best be evaluated when the steps preceding the onset of illness are examined separately. Different environmental, host-related, and strain-related factors are important during the different steps in the pathogenesis of invasive disease: exposure to a carrier, transmission of infection, and development of invasive disease. Determination of individual risk factors is also helpful in characterizing risk factors for epidemic disease, although assessment of the role of several key factors is hampered by insufficient longitudinal and population-based data.

Morbidity and mortality from meningococcal disease can be significantly reduced by using currently available vaccines in groups at high risk for disease, particularly during epidemics. Therefore, defining the risk factors for infection and continuing surveillance for disease remain important public health goals for the control of meningococcal disease. However, polysaccharide vaccines are ineffective in young children, and the duration of protection is limited in children vaccinated at 1 to 4 years of age (44). Therefore, development of polysaccharide-conjugate vaccines should be given high priority for the control of epidemic disease by routine immunization of young children. A protective serogroup A-conjugate vaccine might be effective in the Expanded Program of Immunization to interrupt the cyclic epidemics in the African meningitis belt.

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β-Lactamase Plasmids and Chromosomally Mediated Antibiotic Resistance in Pathogenic *Neisseria* Species

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The decrease in the susceptibility of *Neisseria gonorrhoeae* isolates to antimicrobial agents has been extensively documented over the past four decades (12, 20, 34, 67, 76). Prior to the isolation of β-lactamase plasmids from gonococci in 1976, the mechanism of resistance to antimicrobial agents was believed to occur only by chromosomal mutation (68, 70). In most areas, chromosomal mutation remains the major resistance mechanism in gonococci. In the 1980s plasmid-mediated resistance to tetracycline was characterized in gonococci for the first time (56). Trends in antimicrobial resistance in *N. gonorrhoeae* have recently been mimicked by other pathogenic *Neisseria* species (8, 47). The possibility that increasing numbers of *N. meningitidis* isolates will acquire plasmid-mediated resistance to penicillin is of great medical concern.

In the present article, the molecular biology of the β-lactamase plasmids in pathogenic *Neisseria* species is reviewed. This includes a discussion of plasmid diversity and structure, as well as the replication and mobilization characteristics of these plasmids. The gonococcal tetracycline resistance plasmids are not addressed, since they are reviewed in an accompanying article (56). Finally, the genetic loci and specific resistances attributed to chromosomal mutation are briefly reviewed.

MOLECULAR BIOLOGY AND EVOLUTION OF β-LACTAMASE PLASMIDS IN *NEISSERIA GONORRHOEAE*

Plasmid Diversity

β-Lactamase-producing isolates of *N. gonorrhoeae* were first isolated in 1976 in the United Kingdom and North America (3, 7, 48, 49, 51). Strains from the United Kingdom were linked epidemiologically to West Africa, required arginine for growth, were susceptible to tetracycline, and carried a 5.1-kilobase-pair (kb) (reported size range, 3.2 to 3.4-megadaltons [MDa]) β-lactamase plasmid, often called African, as well as the 4.2-kb cryptic plasmid (11, 49, 55). Strains in North America were linked to the Far East, were proline auxotrophs or wild type, were often less susceptible to tetracycline, and carried the 4.2-kb cryptic plasmid, a 7.2-kb (reported size ranges of 4.4-4.7-MDa) β-lactamase plasmid, often called Asian, and a 39.2-kb (24.5-MDa) transfer plasmid (11, 49, 55). Molecular analysis and hybridization studies showed that the African and Asian plasmids were identical except for a 2.1-kb fragment missing from the African plasmid (8, 10, 16, 17, 25, 55). Both plasmids produced TEM-1 β-lactamase and carried about 40% of the transposon Tn*2* (26, 55, 61). *N. gonorrhoeae* strains harboring these plasmids have subsequently caused many outbreaks worldwide (54). The host range of these β-lactamase plasmids has been extended in *N. gonorrhoeae* from original observations to include other auxotypes; the transfer plasmid has subsequently become established in isolates with the 5.1-kb plasmid (2, 22, 75).

In 1984, a third β-lactamase (4.9 kb, designated Toronto) plasmid was recovered from strains of *N. gonorrhoeae* isolated in two Canadian provinces (79, 80). Retrospective analysis based on the genetic similarity of the strains established epidemiological linkages between them. Strains carrying the 4.9-kb plasmid were prototrophic, were serovar Bacjk (1B-2), and carried both cryptic and transfer plasmids (81). The Toronto plasmid was derived from the 7.2-kb (Asian) plasmid; however, the 2.3-kb fragment missing from the Toronto plasmid differed from the 2.1-kb fragment missing from the 5.1-kb β-lactamase plasmid (79, 80). Isolates with Toronto plasmids subsequently isolated in Canada belonged to the same auxotype, and more than 80% belonged the same serovar as isolates from the first outbreak (J. R. Dillon, unpublished data).

Two additional β-lactamase plasmids not associated with outbreaks of β-lactamase-producing *N. gonorrhoeae* infection have been described (30, 74). In early 1984, two β-lactamase-producing *N. gonorrhoeae* isolates, one a methionine auxotroph and the other prototrophic, were recovered in the Netherlands (74). These isolates carried cryptic (4.2-kb) and transfer (39.2-kb) plasmids and identical 4.6-kb β-lactamase plasmids. Because one of the isolates was epidemiologically linked to Rio de Janeiro, the 4.6-kb plasmid was designated Rio. The restriction endonuclease maps of the Rio and the Asian plasmids were similar, except that the small *Hin*dIII-*Bam*HI fragment in the Rio plasmid was reported to be 0.1 kb smaller than the equivalent fragment in the Asian plasmid.

The second distinctive β-lactamase plasmid recovered from a single β-lactamase-producing *N. gonorrhoeae* isolate was a 6.6-kb plasmid, isolated in Nimes, France, which has been called the Nimes plasmid (32). This plasmid was derived from the 5.1-kb African plasmid through a 1.2-kb insertion in the 2.4-kb *Bam*HI fragment (30).

A comparison of the five different β-lactamase-producing plasmids characterized in *N. gonorrhoeae* isolates is shown in Fig. 1. The gel depicts *Hin*fI digests of the five plasmids; all carried a 1.1-kb *Hin*fI fragment associated with Tn*2* sequences. In comparing the Rio plasmid pGO4717 with the Toronto plasmid pJD7, the 0.1-kb deletion previously reported on the *Bam*HI-*Hin*dIII fragment was not observed (74). We therefore concluded that the Rio and Toronto plasmids may be similar; however, only deoxyribonucleic acid (DNA) sequencing will definitively pinpoint minor differences. It thus appears that Toronto and Rio plasmids evolved independently in several countries at about the same time.

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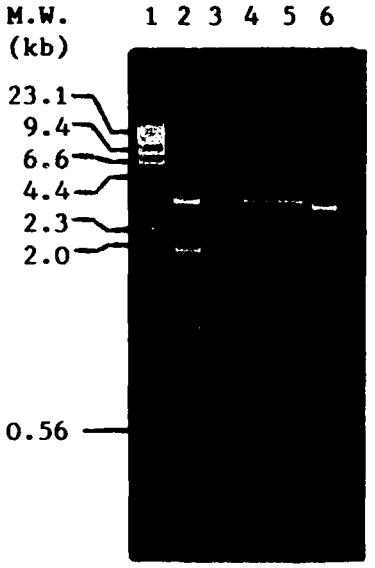

FIG. 1. Restriction endonuclease analysis of pJD4, pJD5, pJD7, pGO4717, and pGF1. Lanes: 1, *Hin*dIII-digested λ DNA as marker; 2, plasmid pJD4 digested with *Hin*fI; 3, plasmid pJD5 digested with *Hin*fI; 4, plasmid pJD7 digested with *Hin*fI; 5, plasmid pGO4717 (Rio type, obtained from J. D. A. van Embden, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands) digested with *Hin*fI; 6, plasmid pGF1 (Nimes type, obtained from A. Gouby and M. Ramuz, Centre Hospitalier Regional et Universitaire de Nimes, Nimes, France) digested with *Hin*fI. M.W., Molecular size.

Site of Deletion in African Plasmids

There has been considerable controversy over the exact position of the 2.1-kb fragment missing in the 5.1-kb African plasmid as compared with the 7.2-kb Asian plasmid. In heteroduplex studies with 7.2-kb (pJD4) and 5.1-kb (pJD5) plasmids, Yeung et al. (79, 80) located the 2.1-kb fragment 1.74 kb counterclockwise from the unique *Pst*I site through the *Xba*I site (Fig. 2). Dickgiesser et al. (16, 17), also mapped the deletion as 1.75 kb from the unique *Pst*I site, except that the deletion was mapped clockwise from this site and was not oriented with respect to the unique *Ava*I and *Pva*II sites present on both the 7.2- and 5.1-kb plasmids. McNicol et al. (45) mapped the 2.1-kb fragment >1.75 kb from the *Pst*I site and within approximately 200 base pairs (bp) of the *Ava*II site. Observations by Aalen et al. (1) are in agreement with the findings of Yeung et al. (79, 80), who placed the deleted fragment at least 900 bp from the *Ava*I site.

It has been widely assumed that different gonococcal β-lactamase plasmids of similar size are identical, and a number of different plasmids of the same size have been studied (1, 10, 14, 17, 45, 66). A comparison of interplasmid differences has not been undertaken, nor have comparative DNA sequencing studies been done to definitively identify the position of the 2.1-kb deletion or other variations.

Minimal Regions Required for Replication of β-Lactamase Plasmids

The 7.2-kb (pJD4) plasmid of *N. gonorrhoeae* has a broad host range and can be mobilized to and replicate in a variety of *Neisseria* species as well as other genera: *Escherichia coli*, *Salmonella minnesota*, *Haemophilus influenza*, *H. parainfluenzae*, *H. ducreyi*, and *N. meningitidis* (10, 21, 29, 31, 33, 70). The host range of the 5.1-kb plasmid has not been as extensively studied as that of the 7.2-kb plasmid.

Contradictory results regarding the DNA fragment responsible for the maintenance and replication of the 7.2-kb plasmid of *N. gonorrhoeae* have been published (35, 46). McNicol et al. (46) cloned both the 2.4-kb and the larger *Bam*HI fragments of a 7.2-kb (p22209) and 5.1-kb (p88557) plasmids of *N. gonorrhoeae* into a pBR322 derivative and found that the essential replication region was located on a 0.8-kb portion of the 2.4-kb *Bam*HI fragment that excluded the β-lactamase (*bla*) gene. Conversely, Johnson (35), who also cloned the *Bam*HI fragments of a 7.2-kb (pGR9091) plasmid into pBR322 or pMB8, reported that the replication region was located on a 3.7-kb *Bam*HI-*Pva*II fragment, which is part of the large *Bam*HI fragment (4.8 kb). Deletions of the *Bam*HI-*Hin*dIII portion of this fragment rendered the recombinant plasmid unable to replicate in a *polA* host and further defined the region essential for replication. This controversy prompted us to further investigate the replication regions of the 7.2-kb (pJD4) and 5.1-kb (pJD5) β-lactamase plasmids (K.-H. Yeung and J. R. Dillon, Plasmid, in press).

The 2.4-kb *Bam*HI fragment with the *bla* gene of Tn*2* was shown to play no role in the replication of the 7.2-kb β-lactamase plasmid in an *E. coli* host (Yeung and Dillon, in press). First, strategies to obtain a mini-β-lactamase plasmid consisting only of the self-ligated 2.4-kb *Bam*HI fragment were unsuccessful. Second, by cloning a 1.2-kb *Hae*II fragment from pACYC184 containing the chloramphenicol resistance determinant into the *Pva*II site of the 7.2-kb plasmid pJD4 and by subsequently deleting the 2.4-kb *Bam*HI fragment (which carried the *bla* gene), a replicon comprising only the 4.8-kb *Bam*HI fragment with the chloramphenicol gene from pACYC184 was created. Thus, the DNA sequences adjacent to the Tn*2* region on the 2.4-kb *Bam*HI fragment are not required for replication of the 7.2-kb plasmid (pJD4).

To define the minimum region required for the autonomous replication of the 7.2-kb plasmid pJD4, we constructed a series of deletion derivatives of pJD4 (79, Yeung and Dillon, in press). The smallest β-lactamase replicon that could be generated was 3.4 kb (designated pJD9), and replication function was attributed to a 1.5-kb region designated a (Fig. 2), which carried a unique *Hin*dIII site and essentially comprised the region encompassed by the 2.1-kb fragment deleted in the 5.1-kb plasmid.

The 7.2-kb β-lactamase plasmid of *N. gonorrhoeae* can replicate and maintain itself in a *polA E. coli* host (35, 46). The DNA sequence of part of replication region a (Fig. 2) indicated that it was structurally similar to other characterized replication regions for the following reasons. (i) Two consensus *dnaA* boxes (5′-TTACACACA-3′, 5′-TTACA CAGA-3′) were identified, indicating that the a region may require the DnaA protein (which induces topological features in the DNA structure and directs the entry of other proteins involved in the replication complex) for replication (63, 81). (ii) The integration host factor of *E. coli* recognizes the consensus sequence C/TAAnnnnTTGATA/T and has recently been shown to be required for the replication of plasmid pSC101 (28). A similar sequence (5′-CAATATCGT GATA-3′) was identified in replication region a of pJD9. The role of the integration host factor in the replication of pJD9 is under study. (iii) The 752-bp sequence of the "a" region was A+T rich (65%), a characteristic of all replication regions. These A+T-rich sequences facilitate strand separa-

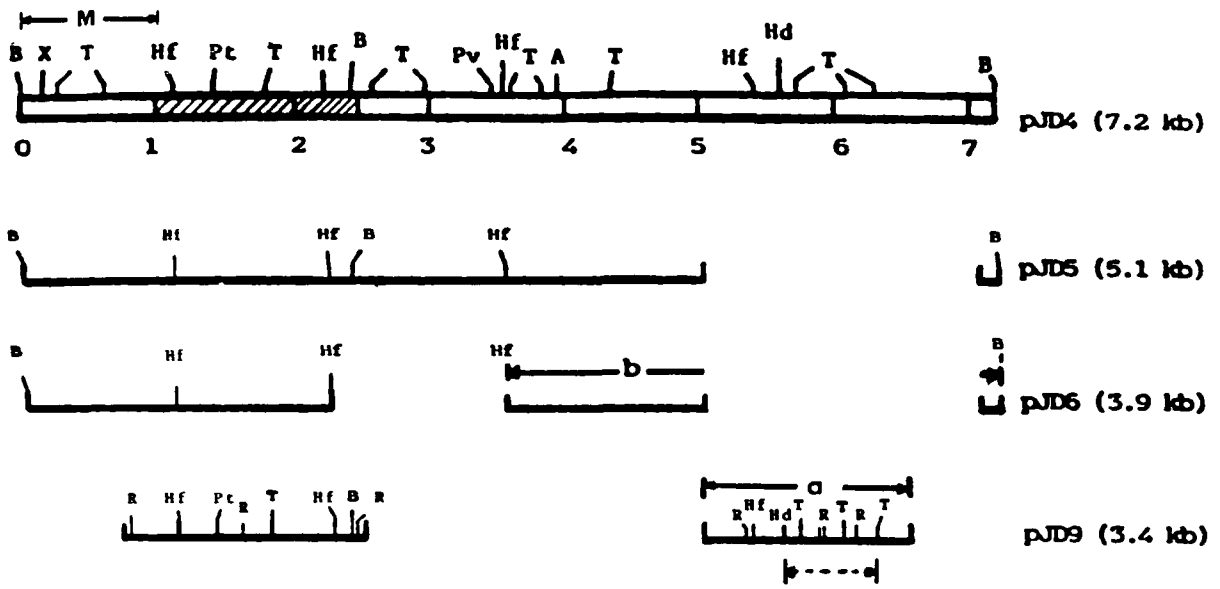

FIG. 2. Physical maps of pJD4, pJD5, pJD6, and pJD9. Symbols: ▨, region homologous to Tn2; ←- - - -→, 752-bp sequence determined from region a of pJD9; M, region required for the mobilization of pJD4; a, replication region a; b, replication region b. Abbreviations: A, *Ava*I; B, *Bam*HI; Hd, *Hin*dIII; Hf, *Hin*fI; Pt, *Pst*I; R, *Rsa*I; T, *Tag*I; X, *Xba*I.

tion during replication (81). (iv) The presence of seven sets of multiple repeats, all A+T rich, suggested that this region had structural characteristics similar to those of plasmids such as pSC101. Replicon-specific proteins interact with repeating sequences and thus regulate replication (81).

Because this replication region (a) spanned the deleted portion of the 5.2-kb β-lactamase plasmid as compared with the 7.2-kb plasmid, we hypothesized that the 5.2-kb plasmid (pJD5) had a different replication region (Yeung and Dillon, in press). Digestion of the 5.2-kb plasmid with *Bam*HI produced two fragments, of 2.7 and 2.4 kb in size. As with the 7.2-kb plasmid, attempts to obtain a miniplasmid comprising only the 2.4-kb *Bam*HI fragment were unsuccessful. However, the size of pJD5 (Fig. 2) was reduced by deleting the *Hin*fI fragment proximal to the *bla* gene to produce pJD6 (3.9 kb). This plasmid could not be further reduced in size. Because the 2.4-kb *Bam*HI fragment alone is not a replicon, the essential region for replication on the 5.1-kb plasmid was tentatively assigned to a 1.5-kb *Hin*fI-*Bam*HI fragment designated replication region b (Fig. 2).

Although there is clear evidence that the 2.4-kb *Bam*HI fragment is not required for the replication of the 7.2-kb β-lactamase plasmid, this conclusion regarding the 5.1-kb plasmid cannot be categorically made. The 1.2-kb *Hae*II fragment of pACYC184 containing the chloramphenicol resistance gene was cloned into the *Pvu*II site of pJD5 (Fig. 2). The 2.4-kb *Bam*HI fragment was then deleted from this plasmid, and ampicillin-susceptible transformants were obtained; however, the clones were unstable, and plasmids with the expected size (3.9 kb) could not be isolated (Yeung and Dillon, in press). Such a construct was stabilized when the pACYC184 origin of replication was also simultaneously cloned with the chloramphenicol resistance gene. We therefore concluded that the 2.4-kb *Bam*HI fragment contributed to the stability of pJD5. Lately, it has come to our attention that a replication region designated *oriI* (1.5 kb) was cloned from a 7.2-kb plasmid (pFA3) and that this region spanned the *Xba*I-*Bam*HI sites and contained part of region b (K. Gilbride and J. Brunton, personal communication). The relationship between region b and *oriI* remains to be established.

Since replication and maintenance properties of plasmids are associated with incompatibility functions (73), the incompatibility relationships between pJD4 and pJD5 and various in vitro-derived deletion derivatives were studied (Yeung and Dillon, in press). These studies showed that the replication regions a and b were different and therefore compatible with one another and incompatible with replicons having the same replication region.

The essential replication region on the 5.1-kb β-lactamase plasmid is also present on the 7.2-kb β-lactamase plasmid. In the 7.2-kb plasmid, region b was interrupted by the apparent insertion of region a. The contribution of region a when both regions are present is unknown, but the insertion of region a may render the b replicon nonfunctional.

Plasmids that contain more than one origin of replication are usually large, conjugative plasmids (58). The 7.2-kb β-lactamase plasmid is therefore the first naturally occurring, small, nonconjugative plasmid shown to contain more than one replication region.

Mobilization of β-Lactamase Plasmids

In addition to the 39.2-kb transfer plasmid from *N. gonorrhoeae* and the transfer plasmids from *H. ducreyi*, the 7.2-kb plasmid can be mobilized by the broad-host-range IncP plasmids from *E. coli* to different bacterial species, as well as from *E. coli* into *N. gonorrhoeae* (31, 45, 52). We have established that in addition to the IncP plasmid RP4, three other conjugative plasmids, R124 (IncFIV), R100 (IncFII), and pBG791 (IncIα), mobilized the 7.2-kb β-lactamase producing plasmid between *E. coli* recipient strains (K.-H. Yeung and J. R. Dillon, unpublished data). These results contrast with those of other reports, in which IncIα (R64drd11) and IncFII (R100drd1) plasmids did not mobilize the 7.2-kb plasmid (31).

Studies to locate the mobilization region of the 7.2-kb β-lactamase plasmids have been characterized by significant

variations in experimental design. Tenover et al. (72) found that a chimeric plasmid comprising the 7.2-kb plasmid (with a deleted 1.9-kb *Hin*fI fragment) and sequences from the transfer and cryptic plasmids could not be mobilized to *E. coli* or *N. gonorrhoeae* and was deficient in the production of a 16-kDa protein. By contrast, in studies based on formation of relaxation complexes, McNicol et al. (45) placed *oriT* on 1.7-kb *Bam*HI-*Hin*dIII fragments of the 7.2- and 5.1-kb plasmids p2209 and p88557.

Further experiments to identify the region required for the mobilization of pJD4 were completed by cloning *Bam*HI or *Bam*HI-*Hin*dIII fragments (3.1, 2.4, and 1.7 kb) of pJD4 (Fig. 2) into pACYC184 (K.-H. Yeung and J. R. Dillon, unpublished data). Mating experiments were performed to detect which of the recombinant plasmids could be mobilized by pBG791 (an IncIα plasmid) (5) at a frequency characteristic of pJD4 (10^{-4}); the mobilization frequency of pACYC184 was 10^{-6} to 10^{-8}. Only the recombinant plasmid containing the 2.4-kb *Bam*HI fragment of pJD4 was mobilized by pBG791 at a frequency (10^{-4}) similar to that of pJD4. Therefore, the 2.4-kb *Bam*HI fragment of pJD4 carried functions required for the mobilization of this plasmid by pBG791. Because this fragment contained a 1.4-kb portion of Tn*2*, it was concluded that the region required for the mobilization of pJD4 was located within the remaining 1-kb fragment, which was designated region M (Fig. 2).

The DNA sequence spanning the M region has recently been published (61). We analyzed this sequence for possible similarities to sequences characteristic of mobilization regions on other plasmids. The M region contained two sequences, 5′-ACCCAGT-3′ and 5′-TGGCTTA-3′, which were found within the origin of transfer (*oriT*) region of ColE1 (15, 78): the later sequence has been recognized as the *nic* site for ColE1. The M region also carried the recognition sequence 5′-AAGCGG-3′ for the n′ protein, which is part of the primosome complex found in the *oriT* regions of F and ColE1 (78). These sequence similarities provide some evidence that the mechanism of mobilization of pJD4 may be similar to that of ColE1, a conclusion also drawn by Guiney and Ito (31) on the basis of the formation of protein-DNA relaxation complexes.

It has recently been reported that the integration host factor was required for the transfer of R100 (44). The integration host factor recognizes the consensus sequence 5′-C/TAANNNNTTGATA/T-3′ (28, 40, 44). Four such consensus sequences, 5′-AAACACGTTGATT-3′, 5′-TAATA CCTAGATT-3′, 5′-AAATTTTTTGATT-3′, and 5′-AAAA TTTTTGATT-3′, with only one mismatch, were found in the M region. In addition, the M region also contained a pair of inverted repeats, 5′-AGCAATCTCTAG-3′ and 5′-CTCGT GATTGCT-3′, similar to inverted repeat sequences (5′-AGCAATCGGC-3′ and 5′-CTGAGATTGCT-3′) which were found in the *oriT* region of R100 (44).

Other evidence suggests that the 7.2-kb plasmid may have two origins of transfer. This conclusion is based on the observations that the IncIα plasmid pBG791 could mobilize deletion variants (e.g., pJD9 [Fig. 2]) of the 7.2-kb plasmid lacking region M. The second mobilization region most probably occurred within region a, a region which also contained a replication region. The second mobilization region may not have been detected in the cloning experiments because the plasmid-specific mobilization protein was truncated or deleted. This finding supports a previous observation that *oriT* was located on the 1.7-kb *Bam*HI-*Hin*dIII fragment of the 7.2-kb plasmid (45). The possibility that the 7.2-kb plasmid has two origins of transfer is also indirectly supported by the observations of Ikeda et al. (33), who found that 3 of 20 clinical isolates of *N. gonorrhoeae* containing both the 39.2- and 7.2-kb plasmids were able to transfer the 7.2-kb plasmid to *N. meningitidis*. In the same study, gonococcal isolates which were unable to mobilize the 7.2-kb plasmid into *N. meningitidis* were able to mobilize the 7.2-kb plasmid into *E. coli*. These observations can be explained only if there are two mechanisms by which gonococcal conjugative plasmids mobilized the 7.2-kb β-lactamase plasmid of *N. gonorrhoeae*; one of the mechanisms was nonfunctional in instances in which the 7.2-kb plasmid was not mobilized into *N. meningitidis*.

Origin of Gonococcal β-Lactamase Plasmids

Gonococcal β-lactamase plasmids were homologous to a variety of plasmids isolated from *H. influenzae*, *H. parainfluenzae*, and *H. ducreyi* (Fig. 2) (8, 14). The 7.0- and 5.7-MDa β-lactamase plasmids from *H. ducreyi* were identical to the 7.2- and 5.2-kb gonococcal plasmids, except that the *H. ducreyi* plasmids carried a complete Tn*2* sequence and an approximately 273-bp segment adjacent to the left inverted repeat of Tn*2* (8, 14). *N. gonorrhoeae* 7.2- and 5.2-kb plasmids lacked a Tn*2* segment of 3,298 bp comprising the left inverted repeat and its proximal sequences (14). The 7.2- and 5.1-kb plasmids from *N. gonorrhoeae* have a G+C content characteristic of *Haemophilus* chromosomal DNA (40%), not *Neisseria* chromosomal DNA, further supporting their non-*Neisseria* origins (55).

A 2.5-MDa plasmid from *H. parainfluenzae*, which did not carry Tn*2* sequences, was shown to be homologous to both *H. ducreyi* and *N. gonorrhoeae* β-lactamase plasmids (9). Chen and Clowes (14) found that the nucleotide sequences of β-lactamase plasmids from *H. ducreyi* (8.6 and 10.5 kb) and *N. gonorrhoeae* (5.1 and 7.2 kb) were consistent with a model which predicted the transposition of the Tn*2* transposon at the target site 5′-TATCT-3′, possibly on an indigenous plasmid of *H. parainfluenzae* (Fig. 3). At some early stage, cytosine was replaced by thymidine (Fig. 3) at 3,773 bp from the left inverted repeat terminus of Tn*2* (14). Divergence in the evolution of this hypothetical plasmid then occurred; because a 2.1-kb fragment (which we have shown to contain replication region a) was inserted, resulting in the formation of another distinctive plasmid. These two hypothetical *Haemophilus* plasmids probably evolved independently; analysis of two such plasmids from *H. ducreyi* indicated that each underwent unique point mutations (C to T on pJB1, and G to A on pHD131) (14). These mutations do not appear on the gonococcal β-lactamase plasmids, thereby implying a separate evolutionary pathway. Although one report noted the transfer of a 7.2-kb plasmid from *H. parainfluenzae* to *N. gonorrhoeae*, others have been unable to conjugally transfer plasmids from *Haemophilus* spp. to *N. gonorrhoeae* (8, 70). Sox et al. (66) have observed that the 7.2-kb plasmid underwent deletions when transformed into homogenic gonococci. Some transformants were identical to the 5.1-kb plasmid. Therefore, it seems likely that gonococcal plasmids arose through the transformation of plasmid DNA from *Haemophilus* spp. following the deletion of the left-hand end of Tn*2*. *N. gonorrhoeae* itself then acted as a reservoir for β-lactamase plasmids, transferring them to other *N. gonorrhoeae* and to *Haemophilus* spp. (21, 27, 29).

CHROMOSOMAL RESISTANCE

In retrospective studies of *N. gonorrhoeae* strains isolated in the prepenicillin era (i.e., before 1944), many isolates were

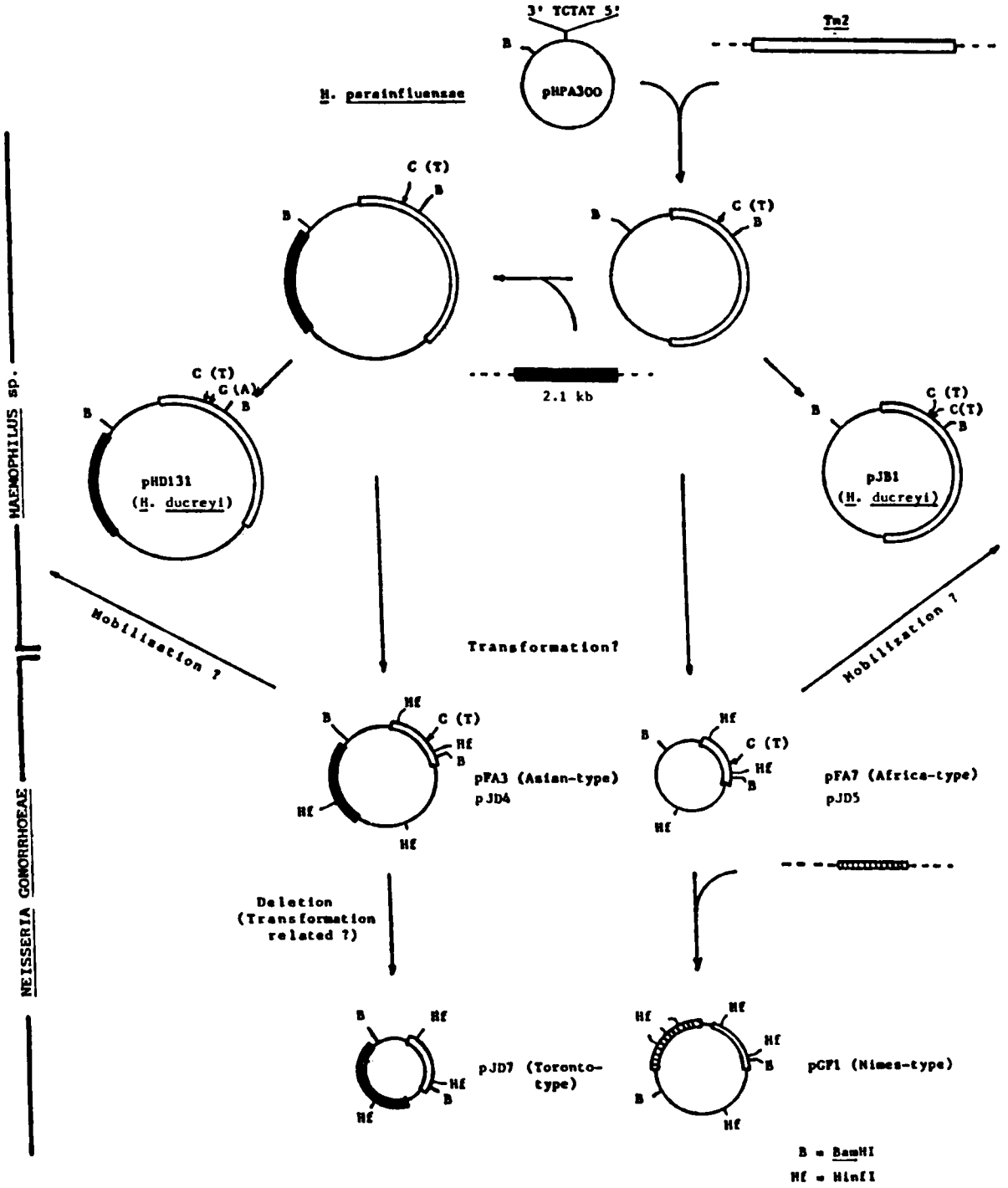

FIG. 3. Evolution of the β-lactamase-producing plasmids in *N. gonorrhoeae*.

found to be susceptible to <0.05 mg of benzylpenicillin per liter, <0.5 mg of tetracycline per liter, and <0.2 mg of spectinomycin per liter. Other isolates were highly (at least 10-fold more) susceptible (12). Since that time, gonococci with reduced susceptibility to antimicrobial agents as a result of chromosomal mutations have increased significantly worldwide (11, 20, 34, 76). Decreased antimicrobial susceptibilities have contributed to increased frequencies of treatment failure and have necessitated repeated revisions of treatment regimens based on specific antimicrobial susceptibility data for a given area (13, 68).

Recently, *N. gonorrhoeae* isolates resistant to penicillin and tetracycline (where resistance is defined as a minimal inhibitory concentration [MIC] of >1.0 μg/ml for surveillance purposes) have been isolated with increasing frequency from certain geographic areas and have contributed to significant treatment failure (53). Chromosomally mediated resistance to a diverse array of antimicrobial agents has

TABLE 1. Mutations contributing to alterations in antimicrobial susceptibility in *N. gonorrhoeae*

Genotype	Phenotype	Biochemical basis	References
penA	Four- to eightfold increase in resistance to β-lactam antibiotics	Lowered affinity for penicillin of penicillin-binding protein 2 (PBP2); shift to PBP1 target; decrease in *o*-acetylation of peptidoglycan	11, 23, 68, 69
mtr	Low-level resistance to several unrelated antimicrobial agents; resistance to dyes, detergents, fatty acids	Altered cell envelope with decreased permeability; increased amounts of 52-kDa outer membrane protein	42, 68, 69
penB, *tem*	Low-level (fourfold over base MIC) resistance to penicillin and tetracycline if *mtr* present	PBPs with reduced affinity for penicillin	23, 24, 68, 69, 77
pem	Enhanced resistance to penicillin expressed by *penA* and *mtr*		77
ampA, *ampB*	Low-level (fourfold) resistance to ampicillin		36
ampC, *ampD*	In combination with *ampA* and *ampB*, fourfold increase in MIC of ampicillin		36
mom	Modifier of *mtr*; phenotypic suppression of *mtr*		64
ery	Low-level resistance to erythromycin		42
env (*env-1*, *env-2*, *env-10*)	Increased susceptibility to antibiotics, fusidic acid, Triton X-100, crystal violet; phenotypic suppression of *mtr* and *penB*	Increased phospholipid-to-protein ratio in outer membranes	41, 62, 63, 68, 69
fus, *tet*, *chl*	Fourfold increase in resistance to tetracycline, chloramphenicol, or fusidic acid	(Ribosomal protein alterations for *tet*, *chl*?)	11, 68, 69
rif	Low-level (fourfold) resistance to rifampin	Structural gene for B subunit of ribonucleic acid polymerase	11, 68, 69
spc, *str*	High-level (1 mg/ml) resistance to either spectinomycin or streptomycin	Structural genes for 30S ribosomal proteins altered	11, 42, 67
vnc	Hypersusceptibility to vancomycin		39
vel	Hypersusceptibility to vancomycin and erythromycin		39

been described: resistance to high levels of spectinomycin and sulfonamides, as well as resistance or reduced susceptibility to β-lactam antibiotics, tetracycline, and erythromycin (11, 65, 68, 70). The prevalence of gonococcal isolates with specific resistances has been associated with a number of other factors including serovar and auxotype of the isolate: race, sexual preference, and gender of the host; isolation site; geographic origin; season; and local antibiotic use (13, 18, 19, 34). Chromosomal resistance to one antibiotic often reduced the efficacy of other unrelated antimicrobial agents by virtue of cross-resistance to them (34, 46, 68).

A number of studies and reviews have elucidated some of the genetic loci responsible for altered susceptibility to a variety of antimicrobial agents (11, 24, 68, 70). These are briefly summarized in Table 1. Some of these mutations act in concert to produce stepwise increases in resistance which are additive. For example, increasing resistance to penicillin can be achieved by the cooperative interaction of the *penA*, *penB*, *mtr* (multiple transformable resistance), and *tem* genes (68). A stepwise increase in resistance to ampicillin was observed through the combined effects of four *amp* alleles, *ampA*, *ampB*, *ampC* and *ampD*, none of which conferred resistance independently (36). Similarly, a combination of *tet*, *penB*, and *mtr* genes produces a 16-fold increase in tetracycline MICs (68). A number of loci (e.g., *env*, *mom*, and *vel*) act to suppress mutations in *mtr* or increase susceptibility to antimicrobial agents such as vancomycin or erythromycin (*env*, *vnc*, and *vel*) (39, 62). Other linked genes (*spc*, *str*, *chl*, *fus*, and *rif* [Table 1]) include loci for resistance to antibiotics which affect ribosomal proteins (11, 68, 70). Mutations leading to the development of resistance to sulfonamides have also been documented. Mutations in the methionine-biosynthetic pathway enhance resistance to sulfonamides, and mutations in the gene encoding dihydropteroic acid synthetase reduce its affinity for sulfonamides (12, 32).

RESISTANCE TO ANTIMICROBIAL AGENTS IN *N. MENINGITIDIS*

The recommended treatment for invasive meningococcemia is penicillin G; chloramphenicol is recommended for patients sensitive to penicillin. Meningococci, until recently, have generally been found to be susceptible to the antimicrobial agents used for primary treatment. In 1983, the first β-lactamase-producing isolate of *N. meningitidis* was re-

ported (21). It was isolated from the genitourinary tract and carried a 7.2-kb plasmid identical to that found in isolates of *N. gonorrhoeae* (21). In 1987, two β-lactamase-producing isolates of *N. meningitidis* were cultured from the cerebrospinal fluid of two patients with meningitis (6). β-Lactamase plasmids can be transferred in vitro from *N. gonorrhoeae* to *N. meningitidis* (35). These recent results suggest that the recovery of meningococci with plasmid-mediated resistance to penicillin may cease to be a rare event.

In addition to plasmid-mediated resistance to penicillin, several reports have recently documented the appearance of low-level resistance to penicillin (MIC > 0.1 μg/ml) in meningococci (6, 47, 60, 71). These isolates were found to have a penicillin-binding protein 3 with a decreased affinity for penicillin (47).

Meningococcal resistance to sulfonamides (variously defined as MICs > 10 to 100 μg/ml), antimicrobial agents used for both prophylaxis and, in some areas, treatment, has been well documented (4, 50). There is evidence that resistance to sulfonamides has not increased recently and may have decreased in incidence in certain areas (4).

Recently, urethral and pharyngeal isolates of *N. meningitidis*, associated with asymptomatic carriage and expressing high-level tetracycline resistance, have been isolated. Plasmid analysis and DNA probe tests indicated that these strains carried a 25.2-MDa plasmid carrying the *tetM* resistance determinant. In vitro studies have also demonstrated that the 25.2-MDa plasmids in *N. gonorrhoeae*, *Kingella denitrificans*, and *Eikenella corrodens* could transfer the plasmid to *N. meningitidis* serogroups A, B, C, E, 29E, X, Z, and W135, as well as other commensal *Neisseria* species (57).

It appears that pharyngeal and urethral microorganisms, including commensal *Neisseria* species, could serve as a reservoir for the transmission of the 25.2-MDa plasmid, the *tetM* determinant alone, or other plasmids, such as the β-lactamase plasmids, to pathogenic species such as *N. meningitidis* (37, 38, 57, 59). Interestingly, in a 1979 Canadian survey of the susceptibility of 827 *N. meningitidis* isolates to penicillin (J. R. Dillon and F. A. Ashton, unpublished data), 29 of the isolates exhibited reduced susceptibility to penicillin (MICs, 0.25 to 0.5 μg/ml). Only 14 of the 29 isolates were documented as to isolation site, and all 14 were pharyngeal in origin.

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Vaccines for Prevention of Meningococcal Disease

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Neisseria meningitidis is the second leading cause of bacterial meningitis in the United States and is the leading cause in many other countries. In the United States there are 3,000 to 4,000 cases of bacterial meningitis per year, for an incidence of about 1/100,000; *N. meningitidis* is responsible for a number of outbreaks each year. Control of these outbreaks and the severe epidemics that have occurred in many countries required the development of vaccines to prevent meningococcal meningitis.

N. meningitidis is divided into 12 serogroups based on the immunological specificity of their capsular polysaccharides (PSs): A, B, C, H, I, K, L, X, Y, Z, 29E, and W135 (35). Approximately 90% of meningococcal disease worldwide is caused by serogroups A, B, and C. Group A is now rare in the United States, but it is the major meningococcal pathogen in the "meningitis belt" of Africa and a number of Asian countries. In the United States, groups B and C are currently responsible for about 50 and 35% of meningococcal disease, respectively, with groups Y and W135 accounting for the remainder.

For epidemiological purposes, meningococcal strains are now identified by their serogroup, serotype, and subtype, e.g., B:15:P1.16. The disease-associated serogroups have been subdivided into over 20 different serotypes (12) based upon immunological differences in the class 2 and class 3 major outer membrane proteins (OMPs). These proteins are the meningococcal porins, having molecular weights, as estimated on sodium dodecyl sulfate-polyacrylamide gels, of between 34,000 and 40,000. The strains are further subtyped by the immunological specificity of the approximately 46-kilodalton class 1 OMPs. Class 1 proteins are shared by a number of different serotypes, and approximately 10 class 1 subtypes have been identified to date. As will be shown below, both class 1 and class 2 proteins have importance as potential vaccine candidates.

Protection against meningococcal disease has been correlated with the presence of bactericidal antibodies (8). The peak incidence of disease occurs in children under 1 year of age; as a group, they have few or no bactericidal antibodies. In addition, the high susceptibility of individuals with an inherited deficiency of one of the terminal complement components (C5, C6, C7, or C8) for invasive meningococcal disease strongly implicates the importance of bactericidal activity in host defense against these organisms (30). For these reasons, measurement of the bactericidal activity in serum provides an accurate indication of the resistance of an individual to meningococcal disease.

The development and clinical evaluation of meningococcal PS vaccines have been reviewed previously (8, 13, 31), as have the initial studies of the use of protein vaccines for group B disease (8). The present review will therefore concentrate on recent studies, recommendations for use of the PS vaccines, and the current status of a group B vaccine.

PS VACCINES

Immunization against *N. meningitidis* became a reality with the accomplishments of Gotschlich et al. in the late 1960s (14). The capsular PS vaccines developed by Gotschlich et al. were among the first chemically pure bacterial vaccines. The group C vaccine was used to prevent the severe outbreaks of group C meningococcal disease in the U.S. armed forces.

The molecular weight of the PS has been correlated with its immunogenicity (15). In early attempts in the 1940s to prepare purified group A meningococcal PS vaccines, the PS was found to be almost nonimmunogenic (20). Later studies by Gotschlich et al. (15) showed that the early failures were due to degradation of the PS. Hence, the PSs must be of high molecular weight as measured by gel filtration on Sepharose 4B or 2B to be included in the vaccine.

Group A PS

Efficacy of the group A vaccine was shown through a series of field trails in Africa and Finland in the early 1970s (26, 38). These trials indicated that the PS was approximately 90% effective in controlling epidemic group A disease. Protection in the Finnish trial began at 6 months of age (26). The duration of protection in the epidemic and hyperendemic areas of Africa was short in young children (28). Reingold et al. studied the duration of protection in young people, ranging from 6-month-old children to university-age students, against clinical group A meningococcal disease in Burkina Faso, Upper Volta (28). The individuals were vaccinated with an A/C bivalent vaccine. By using case-control studies, the efficacy was found to be 100, 74, and 67% for 1, 2, and 3 years after vaccination in individuals over 4 years of age at immunization, compared with 85, 52, and 8%, respectively, for those under 4 years of age. The rapid fall in protection in the younger children is probably related to the poorer response of young children and to the observation that malaria, endemic in western Africa, interferes both with the immune response to the PSs and with the persistence of antibodies (39).

The decline in protection has been correlated with a decline in group A PS antibodies. Kayhty et al. (21) found that the antibody levels in children who were vaccinated at less than 4 years of age were not different from those in unvaccinated controls 3 years postimmunization.

Group C PS

The group C PS was first shown to be efficacious in U.S. military recruits (1, 31), in whom it virtually eliminated group C meningococcal disease. The group C PS was later found to be effective in preventing disease in children over 2 years of age in an efficacy trial in Brazil (31). The duration of protection was not examined in the Brazilian trial.

The group C PS is a homopolymer of sialic acid-linked α2-9 and is found in two variant forms. Approximately 15% of group C clinical isolates elaborate an *O*-acetyl-negative PS, and the remainder elaborate an *O*-acetyl-positive polysaccharide. Studies were conducted in children and infants to compare the immunogenicity of the two variant PSs (27). Each of the PSs stimulated equivalent responses. The current PS vaccine contains the *O*-acetyl-positive PS.

Recent studies have been conducted to examine the antibody response of young children and infants to the ACYW135 tetravalent vaccine (22, 27). Lepow et al. (22) found that a greater percentage of children between 2 and 5 years of age responded to the Y and W135 PSs than responded to the A and C PSs. An 80% or better response was found to all four PSs in children aged 9 years or older. A problem with the PS vaccine in children aged 8 years and under was the rapid fall in antibody levels, 70 to 80% 1 year after vaccination.

Peltola et al. (27) examined the immune response of infants aged 6 to 24 months to the tetravalent vaccine given at 30 μg per PS. Children under 1 year of age at immunization received a second injection 3 months later. Over 90% of the children responded with bactericidal antibodies to group A. The response to the other PSs was more age dependent, with 80 to 100% of the 18- to 23-month-old children and 40 to 50% of the younger children responding to the PSs. Children under 1 year of age had a rapid decline in bactericidal antibodies, and 1 year later, levels were not different from those in nonvaccinated children of the same age. Peltola et al. recommended that children under 1 year receive two injections 3 months apart. They thought that routine immunization would be justified if an effective group B vaccine component could be added.

Recommendations

The Immunization Practices Advisory Committee of the U.S. Public Health Service has made recommendations for the use of the meningococcal PS vaccines (5). The A, C, AC, and ACYW135 PS formulations are currently licensed in the United States, and recommended immunization is a single 0.5-ml intramuscular injection of vaccine containing 50 μg of each PS. The vaccine is presently available in the United States from Connaught Laboratories and in Europe from Smith Kline-RIT and Institute Merieux.

Routine vaccination of the civilian population in industrialized countries is not currently recommended, because (i) the risk of infection is low, (ii) a vaccine against group B is not available, and (iii) most of the endemic disease occurs in young children. Vaccination is advised to control outbreaks due to meningococcal serogroups covered by the vaccine. Routine vaccination is recommended for travelers to countries recognized as having hyperendemic with periodic epidemic meningococcal disease, such as the meningitis belt of Africa, or recent epidemic disease, such as Nepal and Saudi Arabia.

Vaccination of individuals with a deficiency in one of the terminal complement components or with properdin deficiency may be effective in preventing disease. Patients with deficiencies in C5, C6, C7, or C8 have a prolonged susceptibility to meningococcal disease and often develop the disease later in life; the median age at the first episode is 17 years (30). The low mortality rate (4%) associated with such infections suggests that the presence of antibodies that can promote opsonophagocytosis is important in such individuals. Therefore, it follows that vaccination could provide some protection.

Normal properdin function appears to be necessary for resistance to meningococcal disease. Properdin promotes bacterial killing through activation of the alternative pathway by stabilizing the C3 convertase, C3bBb (33). Many individuals with meningococcal disease associated with properdin deficiency are young children, who would not be expected to have developed protective antibodies (33). Vaccination of properdin-deficient individuals with the meningococcal PS vaccines has been shown to induce antibodies that cause meningococcal killing through the classical complement pathway (6).

Conjugate Vaccines

For meningococcal PS vaccines to elicit antibodies in young children, they will have to be conjugated to protein carriers. The duration of protection in young children is relatively short, and the antibody response is not boostable as is the case with T-cell-dependent antigens. Meningococcal A, B, and C PSs and oligosaccharides have been covalently attached to tetanus toxoid (2, 3, 18). Jennings and Lugowski (18) produced conjugates of the A, B, and C PSs by periodate oxidation and direct attachment through the lysines in the tetanus toxoid. In addition, they prepared oligosaccharides from the group C PS and linked them through a spacer molecule to bovine serum albumin. These conjugates were used to hyperimmunize mice. All except the B conjugate were highly immunogenic. Beuvery et al. (2, 3) produced group A and group C PS conjugates by attachment to tetanus toxoid. These conjugates were compared with the native PSs for their immunogenicity in mice. The native PSs were essentially nonimmunogenic when administered in two doses 10 weeks apart. In contrast, a single injection of the conjugates elicited high antibody levels. A second injection at week 10 elicited a booster response, typical of a T-cell-dependent response. Adsorption of the conjugates to aluminum phosphate significantly increased the primary immune response, but failed to have an effect on the booster response (2, 3). Thus, conjugation of the A and C PSs to a protein carrier converted the PSs from T-cell-independent to T-cell-dependent antigens.

Meningococcal PS-protein conjugates have not been evaluated to date in humans. However, extensive human studies with *Haemophilus influenzae* type b PS conjugates indicate that conjugates are safe and much more immunogenic in young children than the native PS is (16). A second injection of an *H. influenzae* type b conjugate or of the native PS elicits a strong booster response (16). Whereas the type b PS was not effective in children under 18 to 24 months of age, a diphtheria toxoid conjugate proved to be highly efficacious in infants under 1 year of age (7). Meningococcal PS conjugates may also prove to be highly immunogenic in young infants.

GROUP B MENINGOCOCCAL VACCINES

Group B PS

Although the group B PS, a homopolymer of α2-8-linked sialic acid, appears the logical choice for production of a group B meningococcal vaccine, only limited success has been achieved in animal studies (19, 24). The native PS induces only a transient immunoglobulin M (IgM) response in humans (40). A number of possible explanations have been given for the poor immunogenicity, including sensitivity to neuraminidases and the similarity of the PS to sialic acid moieties in human tissues.

Moreno et al. have attempted to improve the immunogenicity of the group B PS by noncovalently complexing it to OMPs (24) and by adsorbing the complexed vaccine to aluminum hydroxide (25). These vaccines were used to immunize mice and then compared with vaccination with the pure PS. Although the PS was nonimmunogenic, both of the other formulations induced transient increases in IgM anti-

bodies that peaked on day 7. A similar aluminum hydroxide serotype protein group B PS vaccine failed to stimulate measurable increases in group B PS antibodies in human studies (11).

Another approach to preparing immunogenic group B PS vaccines is to covalently link the PS to a protein carrier or to chemically modify the PS. The group B PS conjugates were essentially nonimmunogenic. Jennings et al. made a number of chemical modifications to the PS (19). The only modification that did not abrogate the ability of group B-specific antibodies to recognize the PS was removal of the *N*-acetyl groups and substitution of *N*-propionyl groups.

The N-propionylated PS and its tetanus toxoid conjugate were used to immunize mice by using Freund complete adjuvant in a series of three injections (19). The altered PS alone failed to induce group B PS-reactive antibodies. In contrast, two injections of the conjugate induced antibody levels substantially above background, and the third injection gave a good booster response. Interestingly, the antibody response was mostly IgG. These antibodies were shown to be bactericidal against group B strains of different serotypes (17) but not against group A, C, or W135 strains. Quantitative precipitin experiments indicated that about half of the antibodies induced to the N-propionylated PS were specific for the altered PS (17). Unexpectedly, absorption of the sera with only the N-propionylated PS removed the group B bactericidal antibodies, even though the native PS absorbed all radioimmunoassay-reactive antibodies. Thus, the N-propionylated group B PS mimics a bactericidal epitope on the group B organism. Additional studies of the nature of this epitope are important, and limited adult human studies seem warranted.

Important OMPs

A very promising approach to the development of an effective group B meningococcal vaccine is the use of lipopolysaccharide-depleted OMP vaccines. Such vaccines are prepared from outer membrane fragments depleted of lipopolysaccharide by detergent treatment. The detergent is removed, and the final vaccine is formulated to also contain one or more meningococcal PSs to improve its solubility and immunogenicity.

Meningococcal outer membranes contain a number of major proteins, and most of these proteins are included among those designated as the class 1, 2, 3, 4, and 5 proteins (34). These proteins are often present in the OMP vaccines, which induce bactericidal antibodies in both animals and humans. Studies were therefore done to help define the OMPs responsible for the induction of protective antibodies (4, 32). Saukkonen et al. (32) have used their recently developed infant rat model for meningococcal infection to evaluate the protection afforded by monoclonal antibodies to the class 1 and 3 OMPs. Antibodies against both classes were bactericidal, but only the anti-class 1 protein monoclonal antibodies protected against both blood and cerebrospinal fluid infection by the homologous subtype. In similar studies, Brodeur et al. (4) examined monoclonal antibodies against OMP classes 1, 2, and 5 prepared against a serotype 2b strain for their ability to protect in a mouse model; mucin and hemoglobin were used to achieve infection. In this model none of the monoclonal antibodies prevented bacteremia, and only the class 2 serotype 2b-specific monoclonal antibody significantly reduced lethality. The passive protection studies with monoclonal antibodies, taken as a whole, indicate that the class 1 proteins are strong candidates for an OMP vaccine. These proteins are serosubtype proteins and are shared among meningococcal strains to a greater extent than the class 2 and 3 proteins are.

The antigen specificity of the adult response to a serotype 2a OMP vaccine was examined by immunoblotting (36, 37). Most individuals responded to some of the high-molecular-weight proteins and to the class 1 and 5 proteins. It was particularly noteworthy that those who responded with high bactericidal titers had high antibody levels to the P1.2 class 1 protein. The subclass response was primarily IgG1 and IgG3 (37). The class 1 protein is therefore an important antigen for inclusion in an OMP vaccine.

In immunoblots, antibody binding to the class 2 and 3 proteins is dependent upon renaturation in a dipolar-ionic detergent (23). The postvaccination antibodies bound much better to the class 2 protein when the IgG response to the serotype 2a protein was examined in the presence of the detergent Empigen BB (37) than when the detergent was absent. However, the binding level was quite variable among individuals. The response to the class 1 protein remained highest.

Recent Clinical Studies

A number of clinical studies have been conducted with OMP vaccines; all of them indicate that these vaccines are safe and immunogenic. Most individuals will experience local reactions of redness or tenderness at the injection site, but systemic reactions are minimal (11).

Rosenqvist et al. (29) examined the antibody responses of 57 Norwegian adults to a vaccine containing OMPs of serotypes 2b:P1.2 and 15:P1.16 noncovalently complexed to meningococcal PSs A, C, Y, and W135. Volunteers received one dose of the vaccine containing 52 μg of protein from each serotype, 40 μg of each PS, and 3.5 μg of lipopolysaccharide. Overall, 70% of the vaccinees had immune responses to both serotypes as judged by the enzyme-linked immunosorbent assay and bactericidal assay. The vaccinee responses were equivalent to those of group B meningococcal disease patients, who had mostly B:15:P1.16. The antibody response was predominantly IgG, as we had found in similar studies (11). The majority of the vaccinees had substantial increases in bactericidal antibodies to both serotypes included in the vaccine. Before vaccination, 60% of the individuals had no detectable bactericidal activity against either serotype, and after immunization, only 21 and 24% lacked bactericidal antibodies against types 2b and 15, respectively. A second immunization or use of adjuvants would probably improve the response.

An important observation of Rosenqvist et al. (29) was the excellent correlation ($r = 0.8$) between the IgG levels measured by the enzyme-linked immunosorbent assay and bactericidal titers measured by using human complement. Earlier studies in which baby rabbit serum was used as a source of complement failed to show a correlation between the two assays.

The major target population for a group B vaccine is young children. We have therefore compared the immune responses of adults and children to vaccines consisting of serotype 2a OMP noncovalently complexed with group B meningococcal PS (9). The antibody responses were measured by the enzyme-linked immunosorbent assay with purified serotype 2a outer membrane vesicles and by a bactericidal assay with a group C:2a strain and baby rabbit serum complement. In both assays young children (under 6 years old) had lower antibody responses than did older

children and adults. Over 50% of children over 2 years of age had postimmunization bactericidal titers of 1:8 or greater, compared with only 20% for those under 2 years of age. By comparison, 70 to 80% of adults had a fourfold rise in their bactericidal titer after immunization (8).

In an effort to improve the percentage of young children developing bactericidal antibodies following administration of serotype vaccines, we have evaluated the effect of adsorption of serotype 2b protein vaccines onto aluminum hydroxide. Initial studies in animals indicated that adsorption of the vaccine significantly improved the immune response (9). The experiments have now been extended to studies with adult volunteers (11). Groups of adults were immunized with a serotype 2b-group B PS vaccine with and without aluminum hydroxide adjuvant. The adjuvant improved the antibody response as measured by the enzyme-linked immunosorbent assay and bactericidal assay. Only those who received the first immunization with adjuvant had a booster effect on reimmunization. These studies indicate that aluminum hydroxide or other adjuvants should be used to increase the percentage of children who respond to OMP vaccines.

Efficacy Trials

Trials to estimate the efficacy of OMP vaccines for preventing group B meningococcal disease have been conducted in South Africa and are now in progress in Cuba and Chile. A randomized serotype 2a vaccine trial was carried out in Cape Town, South Africa, in 1981 (10). The vaccine was administered to 2,200 children between 4 months and 6 years of age (mean, 2 years). Another 2,200 received a group A and C PS vaccine. The trial was conducted during a group B serotype 2b epidemic and provided evidence for protection against group B serotype 2 disease, but insufficient numbers of children were immunized to allow for a statistical estimate of efficacy. Results are not yet available for the other trials.

Problems with Serotype Protein Vaccines

One problem with the serotype protein approach to prevention of group B meningococcal disease is the restricted range of protection induced by the serotype and subtype proteins. To achieve broad protection, a vaccine would have to contain OMPs from at least three or four serotypes, and inclusion of this number in a vaccine is feasible.

The serotypes associated with significant levels of disease vary from one geographic region to another, as well as over a period of years. Continued epidemiological surveillance of disease-associated serotypes should be done to produce the optimal vaccine for a given period and location.

Although serotype protein vaccines offer the best immediate approach, there may be alternatives. An ideal meningococcal vaccine would be immunogenic in all age groups and protect against all group B strains and possibly all meningococci. However, such a vaccine can be developed only when there is a much better understanding of the basic mechanisms by which the organism is able to gain entrance into the host, evade host defenses, and cause invasive disease. Alternative antigens that must be examined include the high-molecular-weight, iron-inducible OMPs found in all meningococci. These and other surface proteins, such as stress proteins, are induced or greatly increased under in vivo growth conditions. We know, for instance, that patients respond strongly to some high-molecular-weight OMPs that appear only as very minor proteins when the organisms are grown on the usual laboratory media. Therefore, membrane vaccines should be prepared from organisms induced to elaborate these proteins. Other alternative antigens include fimbriae and lipopolysaccharide-derived oligosaccharides covalently bound to a protein carrier.

In conclusion, the existing meningococcal PS vaccines are safe and effective and should be used to control outbreaks due to serogroups A, C, Y, and W135. In severe group B outbreaks, in which only one or two serotypes are responsible for most cases of the disease, a serotype protein vaccine is likely to be valuable. I am confident that serotype protein vaccines will protect against group B and group C disease, but I am concerned that such protection may not provide sufficient coverage; that is, protection may be too serotype restricted. For broad protection, the vaccine potential of alternative cell surface components must be evaluated. When an effective group B meningococcal vaccine is developed, this vaccine would ideally be combined with the other meningococcal PSs, the *H. influenzae* type b PS, and possibly some of the pneumococcal PSs, because our ultimate goal is to prevent the most common causes of pediatric bacterial meningitis.

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Genetic Mechanisms and Biological Implications of Phase Variation in Pathogenic Neisseriae

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A sophisticated strategy used by a number of procaryotic and eucaryotic microorganisms to escape host defense mechanisms is that of varying the expression of their major surface antigens. This immune escape strategy has been nicely documented for African trypanosomes (6) and *Borrelia hermsii* (2). Antigen variation in pathogenic neisseriae leads to another intriguing view of the same phenomenon: the variation of functional properties of the respective surface proteins. There are two classes of proteins in pathogenic neisseriae that undergo antigenic variation, (i) pilin, the major subunit of the pili, and (ii) the opacity-associated proteins (OPAs), previously P.II or class 5 proteins, a family of heat-modifiable outer membrane proteins. Both classes of proteins are substantially immunogenic and have an essential role in bacterial adherence. Conceivably, variation of these proteins not only leads to immunological changes but also gives rise to alterations of their functional (i.e., adhesive) properties. Although little is known about the biological role of antigen variation in *Neisseria* species, extensive molecular genetic investigations performed during the past several years might lead us to gain better insight into this field.

GENETICS OF SURFACE PROTEIN VARIATION

Primary Structure of Pilin and Organization of Pilin Genes

Variation of pilin expression in gonococci relies on a multigene system. The genetics of this system have been studied in greatest detail for *Neisseria gonorrhoeae* MS11 (3, 33); complementary work has been done with *N. gonorrhoeae* P9 and *N. meningitidis* C114 (35, 36). The gonococcal genome usually contains a single structural gene that is responsible for pilin expression (*pilE*); however, two expressed genes are occasionally found (31, 33). The *pilE* locus codes for the production of a pilin precursor (propilin) that carries a seven-amino-acid transport signal at its amino terminus. Posttranslational processing of propilin involves replacement of the short signal peptide by an N-methyl group (21). The signal peptide is linked to an adjacent hydrophobic region which constitutes the amino terminus of mature pilin. Functionally, this hydrophobic region is thought to substitute for the hydrophobic core, typically seen in transport signals but missing in the short signal of propilin. Furthermore, this region has been implicated in the polymerization of pilin into pili. The amino-terminal portion of pilin is conserved among different gonococcal pilin molecules and shows homology with the N-methyl pili of related species (see, e.g., reference 39).

Almost all variations between different gonococcal pilins seem to occur within six short regions, called minicassettes (MCs), located toward the central and carboxy-terminal portions of pilin (14, 15, 54). Of all the minicassettes, MC2 is the most variable and constitutes the immunodominant region of pilin. The short sequences interspersed between the MCs are highly conserved at both the protein and deoxyribonucleic acid (DNA) levels of variant genes (14).

In addition to the expression gene(s), a gonococcal cell contains many silent gene loci (*pilS*). A silent locus carries one or more partial pilin gene copies which are tandemly arranged and connected by repetitive sequences (13). Although these partial gene copies show the same arrangement of variable MCs in comparison with variant expression genes, they are devoid of the conserved amino-terminal coding sequences (13). Silent loci carry several repetitive sequence motifs with counterparts in the expression loci (15). Striking sequence homologies and similarities in the organization of silent and expressed genes are observed between gonococci and meningococci (36).

Variation of Pilin Expression

Changes in the expression of pilin result from recombination of silent gene copies with the expressed gene. In this way, the expressed gene acquires variable minicassettes such that some, but not all, of its minicassettes are replaced by minicassettes from any of the silent loci. This intragenic recombination leads to an immense variability of sequences in the expression genes (14, 15, 54).

Most often, variant propilins produced by an altered expression gene give rise to pili with altered antigenic and adhesive properties (28, 58). Some propilins, however, are processed in an alternative fashion at position 40 (Fig. 1). This alternative cleavage, which seems to depend on the sequence composition in the expressed gene, removes the hydrophobic region of pilin. Consequently, the resulting product (called S pilin) is unable to polymerize; S pilin is instead secreted as a soluble antigen (14). If all the propilin is converted into S pilin, the host cells are of course nonpiliated. Often, though, the propilin of S variants is only partially converted into S pilin, thus giving rise to cells which show weak or intermediate piliation and which produce standard pilin and S pilin at the same time.

Recombination between cassettes of the *pilS6* locus and *pilE* leads to yet another type of variant (L variant) that is characterized by a slightly extended expression locus (P. A. Manning, T. F. Meyer, A. Kaufmann, B.-Y. Reimann, U. Roll, and R. Haas, manuscript in preparation). The extended expression locus codes for a larger pilin which is neither secreted nor assembled into pili (14). Although the biological significance of L variants is unknown, it is interesting that some L variants quickly revert to the piliated phenotype. Moreover, L variants show an increased resistance to various hydrophilic antibiotics, such as penicillin and kanamy-

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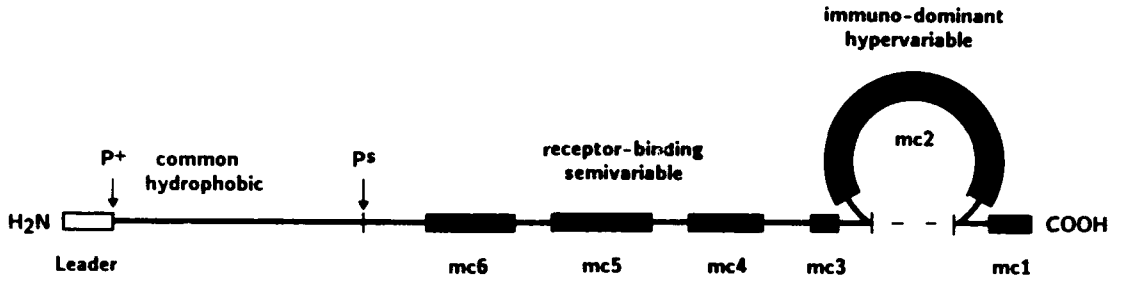

FIG. 1. Primary structure of propilin. Propilin consists of about 160 amino acids plus a seven-amino-acid leader. P^+ and P^s denote alternative processing sites of propilin at positions 1 and 40, respectively. mc1 to mc6 indicate the minicassettes, i.e., the regions which differ in variant pilins and among silent and expressed pilin genes.

cin. It appears that S and L variants account for the majority of nonpiliated phase variants in strain MS11 (14).

Two Mechanisms Account for Pilin Variation

DNA sequence analysis and Southern hybridizations suggest a common mechanistic basis for the generation of P^+, S, and L variants (14; Manning et al., in preparation). Koomey et al. demonstrated that recombination in *pilE* is *recA* dependent (25). Our own studies with another set of *recA* mutants extend this observation for S and L variants. Therefore, the increase in kanamycin resistance observed for L variants (see above), which allows direct quantification of transitions to the L phase, appears to be a suitable tool with which to monitor the frequency of pilin variation in general.

Norlander et al. (34) previously reported a reduction in the frequency of piliation-specific colony morphology changes when cultures are grown in the presence of deoxyribonuclease I (DNase I). Applying L-phase selection, we tested the frequency of phase transitions in the presence or absence of DNase I. The results suggest that in a mid-logarithmic-phase culture, the frequency of P^+ to L-variant transitions is not affected by DNase I; however, in a stationary-phase culture, i.e., when autolysis begins, the frequency of phase transitions eventually rises between 3- and 100-fold, and this increase can be abrogated by the addition of DNase I (B. Y. Reimann, R. Haas, and T. F. Meyer, unpublished data). Similar observations have been made by Seifert et al., who used an engineered marker (*cat*) as a reporter of recombination in *pilE* (46). These authors further demonstrate a substantial decrease in the frequency of phase variation in DNA-uptake-deficient (*dud-1*) mutants (46). Taking into account two other facts, i.e., the tendency of gonococci to undergo spontaneous autolysis (16) and the high competence (47) of gonococci for transformation by species-specific DNA, it seems evident that pilin antigenic variation is driven by transformation-mediated recombination.

Obviously, Southern hybridizations do not allow a distinction to be made between transformation-mediated recombination and the originally proposed mechanism of pilin variation, gene conversion (3, 13–15, 45, 54). To test whether gene conversion accounts for the low frequency of phase transitions seen in the presence of DNase I or in transformation-defective mutants, we selected L variants during stringent exposure to DNase I. Strikingly, Southern hybridization pattern for L variants generated under such conditions does not show the usual nonreciprocal (gene conversion-like) recombination. Instead, such variants have undergone reciprocal recombination involving silent and expressed *pil* genes on the same chromosome (C. P. Gibbs, B. Y. Reimann, R. Haas, A. Kaufmann, and T. F. Meyer, submitted for publication). On the basis of this observation, we conclude that pilin variation occurs by two distinct recombination processes: (i) transformation-mediated recombination (mimicking gene conversion events), and (ii) reciprocal recombination, which occurs at a lower frequency.

In Vivo Significance of DNA Transformation

If transformation is an important factor in pilin phase switching, one should assume that nonpiliated revertible gonococcal variants, although believed to be noncompetent (47), are in fact transformation competent with frequencies comparable to that for piliated gonococci. This can be postulated because switching frequencies from the P^+ to a P^- phase are of the same order of magnitude as the reverse transitions. Recent transformation experiments performed with a gonococcal *recA*::*cat* construct indeed demonstrate that nonpiliated gonococci that are able to revert, i.e., S- and L-phase variants, are highly competent (Gibbs et al., submitted). However, these observations do not prove that transformation has any in vivo significance. This question seems difficult to address directly, since little is known about the stability of DNA from lysed gonococci or about the growth patterns of gonococci during the course of an infection.

Recent DNA sequencing work performed with *iga* genes of various gonococci, however, suggests that horizontal genetic transfer might readily occur under natural conditions (R. Halter, J. Pohlner, and T. F. Meyer, submitted for publication). Sequence comparison of four *iga* genes revealed multiple extended sequence polymorphisms. Some of these polymorphisms are independently conserved in two or three of these *iga* genes. This reveals a mosaiclike genetic composition, which can be explained by efficient horizontal genetic exchange taking place in individuals infected with more than one strain. The recent identification of a specific DNA sequence involved in gonococcal transformation (10) supports the idea that DNA transformation is an essential factor in genetic exchange in gonococci. Of six DNA uptake sequences identified, one was found to be located in the transcriptional terminator of the *iga* gene (10, 37).

Within the *pilE1* locus, no such DNA uptake sequence was detected (10). However, transformation-mediated recombination in this locus (and hence pilin variation) might depend on such DNA uptake sequences that may reside in the vicinity of silent *pil* loci and have thus far escaped our attention.

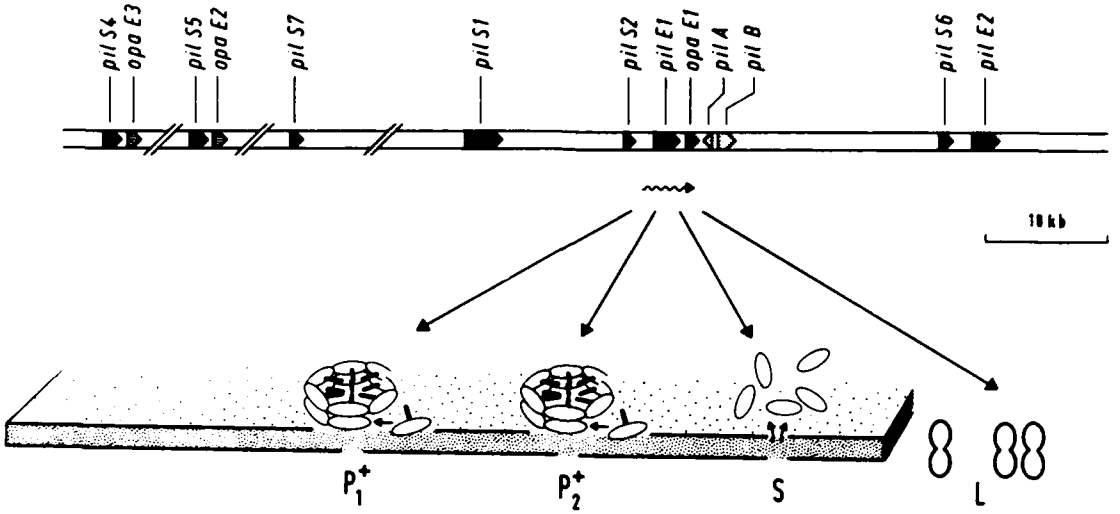

FIG. 2. Organization of pilus gene loci in MS11 and recombination between silent and expression loci. Intragenic recombination in the transcribed pilin gene in *pilE* leads to variant pilin types, i.e., antigenically variant pilins (P_1 and P_2), secreted S pilin (S), and L pilin (L), the precise location of which has not been determined. kb, kilobases.

Pilin and Pilus Expression Mutants

The analysis of pilin variation in gonococci has provided evidence for additional genetic changes, which should be referred to as mutations rather than variations. Two different classes of mutants have been described, deletion mutants and point mutants. Deletion mutants (P_n) have lost the promoter and 5′ coding sequences of the *pilE* structural gene (3, 44). They show a complete loss of pilin-specific expression at both transcriptional and translational levels. The deletion formation seems to depend on the presence of a functional *recA* gene and is virtually irreversible, providing that there is no second active *pilE* locus present in the cell (3; P. F. Sparling, personal communication; our unpublished data). If, however, a cell initially possessed two complete expression loci, one of which became deleted, the second locus might eventually be restored by *pilE* duplication (46), probably via the route of transformation.

The members of the second class of piliation-deficient mutants (P^-) have been characterized as point mutants that can best be isolated from variation-deficient (*recA* mutant) gonococci (25). Such mutants also revert at low frequencies under *recA* conditions. Point or frameshift mutations have been identified at distinct sites in the *pilE* gene, giving rise to assembly-deficient, truncated, and/or unstable pilin (25). One might expect, though, that nonpiliated mutants are affected in accessory genes for gonococcal pilus formation (M. Koomey, personal communication).

Regulation of Pilin Expression

Several lines of evidence suggest that *pilE* is subject not only to DNA rearrangements that lead to the production of altered pilin, but also to regulatory control mechanisms. Apparent sequence homologies exist in the *pilE* promoter region with known DNA-binding sites of the *Klebsiella ntrA* and *nifA* gene products. By *trans*-complementation with *Klebsiella nifA*, activation of pilin expression can be observed in *Escherichia coli* (H. S. Seifert and M. So, personal communication), suggesting that the *pil* gene is controlled by an activator, similar to the NifA activator.

Taha et al. have, in fact, described the isolation of two genes from *N. gonorrhoeae* MS11, *pilA* and *pilB*, that affect transcription of the *pilE* gene in *trans* (56). The two genes are located downstream of the *pilE1* and *opaE1* loci (Fig. 2). The *pilA* product has an activating function and shows at its amino terminus a putative DNA-binding motif. The *pilB* product acts as a repressor. The two genes, which are arranged in diverging orientations, appear to have overlapping regulatory regions. Since *pilA* mutants seem to be nonpermissive in gonococci, they are likely to display multiple effects, comparable to pleiotropic virulence regulators described for other systems (see, e.g., reference 30).

Organization of *opa* Genes

In contrast to the pilin gene system, which includes one or two expression genes and multiple silent partial genes, the *opa* gene system relies on a gene family consisting only of complete genes, each with a functional promoter (49, 50). In *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica*, 12, 3 or 4, and 2 *opa* genes, respectively, have been identified that code for a group of heat-modifiable OPAs (previously referred to as P.II for gonococci or class 5 proteins for meningococci). Most of the *opa* genes carry variant sequences and appear to be constitutively transcribed (49, 50). A genetic linkage exists between some *opaE* and *pil* loci (silent as well as expressed), the meaning of which has not been explained (51) (Fig. 2).

Among gonococcal genes that carry a DNA uptake sequence is the *opaE1* locus (10). This locus was found to undergo recombination with other *opa* loci (49, 51). As in the case of the pilin genes, this process appeared as a nonreciprocal event (8, 49); however, in the light of recent findings, it should probably be attributed to transformation-mediated recombination. Recombination between *opa* genes may occur as an intragenic event, thus giving rise to hybrid genes (8). Although such recombinations can increase the repertoire of a single cell, they do not account for the frequent phase transitions seen for OPA expression in gonococci.

CR Variation: Control of *opa* Expression

An intriguing fact is that all *opa* genes of a cell are transcribed, although not all are translated. This is due to a somewhat peculiar expression control of *opa* genes, which

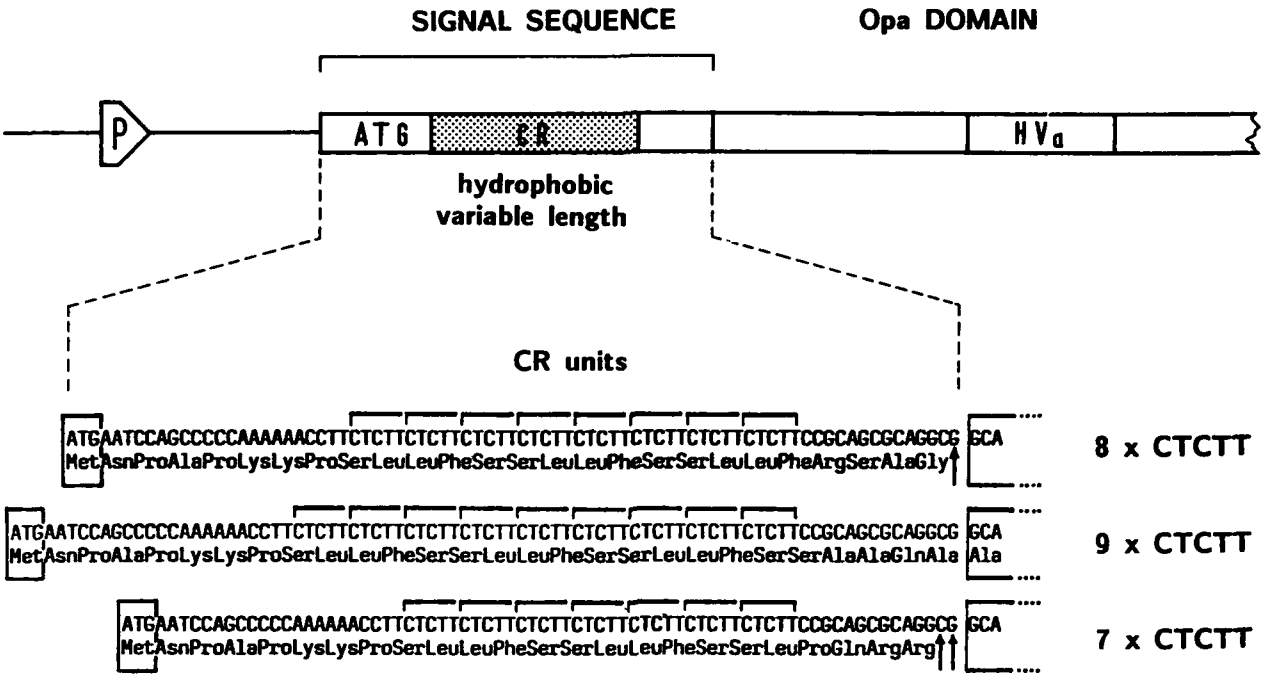

FIG. 3. Location of the coding repeat in the *opa* genes and the control mechanism of *opa* gene expression. The number of CR units determines the reading frame of *opa* genes; if the reading frame is not correct (e.g., eight or nine CR units), no OPA protein will be produced. Changes in the reading frame of *opa* genes are RecA independent and are thought to be due to replicative slippage.

was found to rely on a repetitive sequence (coding repeat [CR]) affecting the translational level (49, 50). The CR codes for the hydrophobic core of the leader peptide of OPA proteins and consists of CTCTT pentamer units (Fig. 3). The molecular principle of *opa* variation is that the number of CR units (CTCTT) is variable in each of the *opa* genes and thereby determines the reading frame of an *opa* gene (49, 50). Depending on the number of CR units (which can range from 7 to 28, apparently without affecting the export functions of the OPA leader), the expression of each of the *opa* genes in a cell can be independently switched on and off. Therefore, the basic repertoire of a single cell is limited to the number of genes present. Only occasionally is this relatively limited repertoire of a single cell altered by recombination events between different *opa* genes, as outlined above (8, 49).

Phase transitions in OPA production are generally more frequent than observed for pilin variation, reaching frequencies of several percent per cell per generation. Because of the particular features of the CR region, we have suggested that CR variation occurs by DNA slippage during chromosome duplication (32, 49). Recently, we (C. P. Gibbs and T. F. Meyer, unpublished results) and others (J. Swanson, personal communication) have shown that CR variation is independent of RecA. Furthermore, Cannon (personal communication) demonstrated by constructing *opa*::*phoA* fusions that CR variation occurs in *E. coli*, suggesting that this process does not need specialized neisserial functions.

BIOLOGICAL SIGNIFICANCE OF ANTIGENIC VARIATION

Pili

Several lines of evidence indicate that gonococci undergo pilin variation during a natural infection: (i) gonococcal isolates from sexual partners often differ in pilus type (63); (ii) pili expressed by gonococcal isolates from different anatomical sites have different antigenic properties (59); and (iii) gonococci isolated from a male urethra after challenge with a piliated strain express pili that differ both antigenically and genetically from pili of the inoculated variant (55). Antigenic variation might be necessary to evade the host immune response and/or as a means to adapt to the particular microenvironments of the infected host, such as the recognition of specific receptors on mucosal surfaces. Indeed, there exists cumulative evidence that pili of variants of a single strain differ not only in physical, chemical, and antigenic properties but also in binding characteristics. Isogenic variants of *N. gonorrhoeae* P9 that produced different pilus types were altered in their ability to adhere to eucaryotic cells. So-called alpha pili bound to human erythrocytes and buccal epithelial cells (28), whereas beta pili preferentially adhered to Chang conjunctiva cells (17). Using isogenic pilin variants of strain MS11, we could show that all piliated variants agglutinated human erythrocytes; some of these variants, however, had lost the ability to adhere to certain epithelial cell lines as well as to cultures of human corneas (58) obtained from different donors. Although all pilin variants produced different pilin types, there was no apparent correlation of the binding capacity with distinct amino acid sequences in the pilin (J. P. M. van Putten, R. Haas, and T. F. Meyer, unpublished data). These observations suggest the existence of different receptors on different human cell types and, furthermore, show that antigenic variation of gonococcal pili is accompanied by changes in the binding characteristics of *N. gonorrhoeae*.

Meningococcal pili also undergo antigenic variation during natural infection. Meningococci isolated from the blood, cerebrospinal fluid, and nasopharynges of individual patients have been found to possess different types of pili (57).

Unfortunately, it is unknown whether these variants of a single strain differ in their adherence to eucaryotic cells. Meningococcal strains (isolated from carriers as well as symptomatic individuals) have been shown to express pilus types with different morphological, antigenic, and binding properties (11, 41, 48, 60). Therefore, antigenic variation might also have functional consequences for meningococci.

The exact nature of the pilus domain(s) mediating adherence to eucaryotic cells is still undefined. Using a monoclonal antibody against a largely conserved sequence (amino acids 69 to 84) of the pilin molecule (42), Rothbard et al. were able to block the adherence of both the homologous and a heterologous strain of *N. gonorrhoeae* to endometrial carcinoma cells, suggesting that this conserved region contains a common receptor-binding domain (40). This finding, however, has not been confirmed by others. Heckels and Virji found that antibodies recognizing a variable domain of the pilin molecule blocked adhesion to Chang conjunctiva cells, whereas antibodies to a common domain had no effect (19). In this context it should be mentioned that at least one of the cross-reacting antibodies used in this study (SM1) was directed against amino acids 49 to 53, a conserved sequence (20) that Rothbard et al. had already found not to be involved in adhesion (40). Interestingly, Swanson et al. reported that the conserved sequence from amino acids 69 to 84 was subject to antigenic variation during natural infection (55). This finding suggests that the sequence from amino acids 69 to 84 is not the (only) domain involved in receptor recognition. It has become apparent that in several Gram-negative species, minor pilus-associated proteins, and not the major pilus subunit, are the mediators of adherence and are responsible for tissue tropisms of an infection (see, e.g., references 26 and 29). Whether gonococci possess pilus-associated proteins, their possible role in adherence, and the relationship between the nature of such proteins and antigenic variation are the subjects of intensive research.

OPAs

The gonococcal OPA (previously P.II) and its OPA counterpart in meningococci (previously class 5 protein) also undergo in vivo antigenic variation during a natural infection. Meningococci isolated from different anatomical sites differ in OPA (38, 57, 63), and variations in gonococcal OPA expression can be found during the menstrual cycle (9, 23). The serological diversity of OPA is relatively limited; gonococci can produce up to seven different OPAs (43), whereas clinical meningococcal isolates originating from a single clone and obtained over a 4-year period have been shown to produce no more than eight different OPAs (1). The biological significance of the variation of OPA expression is still uncertain, although there is evidence that it plays a role in the modulation of adhesive properties of the bacteria. Certain OPAs have been associated with an increased adherence of the bacteria to certain types of epithelial cells, whereas other OPA variants can be associated with the binding to leukocytes (17, 22, 24, 27, 61; J. P. M. van Putten and T. F. Meyer, unpublished results). In addition, gonococcal OPAs that are associated with pronounced colony opacity function as intergonococcal clumping factors (or adhesins) in that they bind to the oligosaccharide part of the lipopolysaccharide of neighboring gonococci (5, 53). Evasion of the host immune defense is, considering the limited repertoire of the variation, probably not the main function of the OPA variation; the variability may rather occur as a response to other stimuli during the course of an infection.

Whether the variability of OPA expression also plays a role in the process after cellular attachment remains to be seen. Immunoelectron microscopy studies indicate that there is no alteration in OPA expression during the adherence and internalization of gonococci into Chang conjunctiva cells (J. F. L. Weel and J. P. M. van Putten, unpublished data).

Identification of Host Cell Receptors

Conclusions drawn from numerous in vivo and in vitro experiments on the binding of neisseriae to different cell types suggest a difference in the density and/or molecular structure of receptor molecules in various tissues (19, 22, 27, 28, 40–42, 48, 57, 58, 60, 61). This diversity in receptor molecules might explain the need for bacteria to produce adhesins, such as pili and OPA, with variable binding specificities. Increasing effort has been made to identify host cell receptors involved in the bacterium-host cell interaction (4, 7, 12, 18, 52, 62). However, in terms of the relationship between antigenic variation, variable binding properties, and the apparent existence of more than a single type of receptor in host tissues, much work remains to be done. The establishment of suitable cell culture systems, the mutagenesis of adhesive properties of *N. gonorrhoeae* and *N. meningitidis*, and the isolation of glycolipids and (glyco)proteins from the membranes of target cells, as well as other strategies, might help to increase our understanding at this point.

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Introduction of Cloned Genes into *Neisseria gonorrhoeae*

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There is considerable knowledge about the genetics and physiology of *Neisseria gonorrhoeae* (see references 6 and 20 for recent reviews). Recent studies with monoclonal antibodies and recombinant deoxyribonucleic acid (DNA), coupled with studies of the immunobiology of various surface antigens, have provided significant advances in our understanding of the molecular pathogenesis of the infections caused by this organism.

Molecular cloning of the DNA encoding various proteins has expedited the identification and characterization of important surface antigens. To date, a wide variety of surface antigens have been cloned in *Escherichia coli* (see reference 33 for a current review). The common thread that underlies these advances is the identification of important genes by cloning and expressing them in *E. coli*. However, many important proteins may be missed by this approach, since *E. coli* may not properly express the genes of interest. In addition, the cell signals that regulate their biosynthesis may not be recognized in *E. coli*. An alternative strategy would be to clone and identify the desired genes directly in *N. gonorrhoeae*. To introduce cloned genes into *N. gonorrhoeae*, a significant amount of research was necessary to identify what genetic tools could be used, what strains could serve as recipients, and what cloning vectors could function in this bacterium.

PLASMIDS OF *N. GONORRHOEAE*

Several different naturally occurring plasmids have been isolated from *N. gonorrhoeae*. Most gonococcal strains contain a 4.2-kilobase cryptic plasmid (19), and a few also contain a 39-kilobase conjugal plasmid (18). Plasmids encoding β-lactamase were first found in *N. gonorrhoeae* in England in 1976 (16), but since then, several related plasmids have been identified elsewhere in strains isolated from patients (9, 12, 32). Recently, plasmids encoding tetracycline resistance have been described (15).

In addition to the naturally occurring plasmids that have been isolated from *N. gonorrhoeae*, researchers in several laboratories have constructed derivatives of some of these plasmids through recombinant DNA techniques. These recombinant plasmids have been exploited for studies of the mechanisms of plasmid DNA uptake during transformation (4) and conjugation (31), as well as for use in cloning gonococcal genes (28).

A bifunctional shuttle vector, pLES2, that is able to replicate in *N. gonorrhoeae* and *E. coli* has been constructed by using one of the naturally occurring β-lactamase plasmids as a base replicon (30). This shuttle vector has the ability to transform *N. gonorrhoeae* at high frequency, when it contains gonococcal DNA (28). To date, this is the only cloning vector that has been used to introduce cloned genes into *N. gonorrhoeae*, but its use is limited by the constraints of the gonococcal transformation and conjugation systems.

GENETIC MECHANISMS OF *N. GONORRHOEAE*

Transformation of *N. gonorrhoeae* was first described by Sparling (23) in 1966. Unlike most bacterial transformation systems, all piliated gonococci are competent for transformation throughout their growth cycle, and phenotypic expression of competence requires only a utilizable energy source and cations (2). Through the use of DNA-mediated transformation, a limited genetic map of *N. gonorrhoeae* has been constructed, although most of the markers included in this map involve ribosomal protein genes (24). Both plasmid and chromosomal DNAs are able to transform *N. gonorrhoeae*, but plasmid transformation is inefficient unless the plasmid contains gonococcal DNA (1). This is because gonococcal DNA is selectively taken up from the environment in a sequence-specific manner (10). Host-mediated restriction can be a barrier to transformation with either form of DNA, although it seems to act more efficiently on plasmid than on chromosomal DNA (25).

The conjugal plasmid present in certain strains of *N. gonorrhoeae* is able to mobilize gonococcal β-lactamase-encoding R factors intraspecifically as well as interspecifically (7, 18). However, this plasmid is unable to mobilize these plasmids efficiently from other bacterial species into *N. gonorrhoeae*, except for *Neisseria cinerea* (8). Piffaretti et al. (17) have shown that pUB307, a derivative of RP1, is able to mobilize gonococcal R factors from *E. coli* to *N. gonorrhoeae*. In addition, this plasmid is able to mobilize the gonococcal shuttle vector pLES2. Through the use of pUB307 and pLES2, it should be possible to use conjugation to mobilize genes cloned in *E. coli* into *N. gonorrhoeae* at high frequencies.

INTRODUCTION OF PLASMIDS INTO *N. GONORRHOEAE*

Considerable attention has been given to the transformation of *N. gonorrhoeae* with plasmids. When plasmid DNA isolated from *N. gonorrhoeae* is reintroduced into it by transformation, the plasmid is linearized during transformation by a nonspecific nuclease (3), yet it retains its ability to transform. In contrast, when the same plasmid is isolated from *E. coli* and then used to transform *N. gonorrhoeae*, it can be restricted in a site-specific manner, and no transformants are obtained (25). Whether or not it is restricted depends on the amount of endonuclease present in the recipient strain, as well as the number of recognition sites that are present on the plasmid (D. C. Stein, unpublished observations).

When *N. gonorrhoeae* is transformed with plasmid DNA that lacks homology with resident plasmids, about 25% of the transformants will contain plasmids of altered size (22). When the recombinant plasmid pLES7, encoding the proline-biosynthetic genes from *N. gonorrhoeae* KH45, was introduced into a proline auxotroph by transformation, the DNA was stably maintained as an extrachromosomal element (29). Furthermore, there was no apparent interaction between the plasmid-encoded DNA and the chromosomal genes. However, in this experiment, the primary selection was for the presence of the plasmid, and then those plasmid-containing cells were screened for the ability to grow in the absence of proline. This experiment inadvertently selected

for intact plasmids, because only cells that possessed plasmid markers were analyzed for the maintenance of gonococcal genes. Nevertheless, these data indicate that plasmid-encoded gonococcal chromosomal DNA can be stably maintained as a plasmid in *N. gonorrhoeae*. When the gene encoding pilin biosynthesis is cloned from *N. gonorrhoeae* PGH3-2 into pLES2, the plasmid is unable to transform *N. gonorrhoeae* PGH3-2 to produce stable transformants, even when properly methylated (Stein, unpublished). This indicates that not all gonococcal genes can be maintained as plasmid-encoded traits.

As an alternative method for introducing recombinant plasmids into *N. gonorrhoeae*, I have cloned a gene encoding a nalidixic acid-resistant form of the gonococcal DNA gyrase onto a plasmid that does not replicate in *N. gonorrhoeae*. When this recombinant plasmid was introduced into *N. gonorrhoeae* by transformation, nalidixic acid-resistant transformants were obtained. When these transformants were screened for the presence of vector sequences by Southern hybridization techniques, no traces were found in the cells. This indicates that plasmid-encoded DNA can interact with chromosomal DNA (Stein, unpublished). This interaction between plasmid and chromosomal sequences occurs even when host-mediated restriction prevents the acquisition of plasmids.

Host-mediated restriction has proven to be an effective barrier in preventing *N. gonorrhoeae* from acquiring plasmids via transformation (25). It is the presence of these enzymes that probably accounts for the deletions seen in plasmids that are introduced into *N. gonorrhoeae* by transformation (22). Although this restriction can be overcome by in vitro methylation (25), the lack of purified methylases has precluded their use in modifying DNA to allow for successful transformation. Host-mediated restriction does not seem to be a barrier to genetic transfer of plasmid DNA via conjugation between different *Neisseria* strains (26). By using RP1 derivatives, it should be possible to introduce any gene, cloned in pLES2, from *E. coli* into *N. gonorrhoeae* by conjugation.

FUTURE STRATEGIES

Researchers in many laboratories would like to study the role of certain cellular components in the disease process by constructing mutants that lack these factors and then using the mutants in specific test systems. Chemical mutagenesis of *N. gonorrhoeae* is inefficient because (i) the dose of most mutagens required to produce a mutation is identical to the lethal dose (5), and (ii) multiple mutations may occur. This has forced researchers in most laboratories to study the genes of interest by cloning and characterizing them in *E. coli*. An alternative strategy would be to apply the techniques of in vitro mutagenesis to the cloned genes and then introduce these mutations into the gonococcal chromosome. Koomey et al. (13, 14) have introduced two different cloned genes into the gonococcal chromosome by transformation-mediated marker rescue. In both cases, they inserted a selectable marker (*bla*) into the gene they were trying to mutagenize. One possible problem with this strategy is that DNA must be inserted into the gene under study. In many cases, the insertion of a foreign gene into the chromosome may block or interfere with the natural processes that regulate the genes expression.

Described below is one strategy that might be applied for the in vivo construction of mutants of *N. gonorrhoeae*. Gonococcal DNA that has been inserted into plasmids incapable of replication in *N. gonorrhoeae* is still able to transform *N. gonorrhoeae*. In addition, when this DNA transforms *N. gonorrhoeae*, the cloned DNA recombines with the chromosome to produce stable transformants. This recombination event happens even if the DNA has had small alterations in its coding sequence, and it also occurs in the presence of host-mediated restriction (Stein, unpublished). By altering cloned DNA through in vitro mutagenesis techniques before introducing the DNA into *N. gonorrhoeae* by transformation, it should be possible to introduce precise mutations into the gonococcal chromosome. This procedure is limited only by our ability to select for the desired transformation event.

TABLE 1. Transformation of FA5100 with plasmid-encoded chromosomal genes

DNA added[a]	No. of transformants generated[b]		
	Nal[c]	L8[d]	Nal[r] + L8[e]
None	$<4 \times 10^{-9}$	$<4 \times 10^{-9}$	NT[f]
pSY6	3.8×10^{-9}	$<4 \times 10^{-9}$	$<2.6 \times 10^{-5}$
pL81	$<4 \times 10^{-9}$	1.6×10^{-3}	NT
pL81 + pSY6	NT	1.6×10^{-3}	6.5×10^{-1}

[a] Approximately 1 ng of plasmid DNA was added for each transformation.
[b] Transformants were generated by adding DNA to ca. 5×10^7 cells and allowing cells to express transforming DNA for 6 h before plating on GCK agar.
[c] Nalidixic acid-resistant transformants were detected by plating cells on GCK agar plus 1 μg of nalidixic acid per ml.
[d] L8 reactive cells were those that reacted with monoclonal antibody 2-1-L8 under the conditions described by Stein et al. (27).
[e] These are nalidixic acid-resistant colonies that reacted with monoclonal antibody 2-1-L8.
[f] NT, Not tested.

A DNA fragment that is able to complement a lipooligosaccharide-defective strain (11, 21) of *N. gonorrhoeae*, FA5100, has been cloned in the cosmid cloning vector, pHC79. When this plasmid (pL81) is introduced into FA5100, it is able to correct the defect and allow for the synthesis of parental LOS (E. F. Petricoin and D. C. Stein, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D187, p. 102). Positive transformants were identified by blotting transformed cells onto nitrocellulose and determining which ones reacted with a specific monoclonal antibody. The best transformation frequencies seen in this system approached 1% (Stein, unpublished), and so several hundred colonies had to be analyzed before a single transformant with the altered trait could be isolated. It seemed probable that if a congression system could be worked out for *N. gonorrhoeae*, it would dramatically increase the likelihood of isolating the desired event.

The data presented in Table 1 illustrate how two cloned genes have been used in a congression experiment to introduce specific DNA fragments into the gonococcal chromosome, even in the absence of the ability to select directly for one of the fragments. This experiment takes advantage of the fact that a gonococcal cell is able to take up more than one piece of DNA. Plasmid pSY6, a plasmid encoding a nalidixic acid resistance gene, is able to transform *N. gonorrhoeae* FA5100 to generate nalidixic acid-resistant transformants. When the concentration of pSY6 used to transform *N. gonorrhoeae* is limiting and the concentration of a second plasmid encoding the nonselective trait is saturating, 65% of the nalidixic acid-resistant transformants also acquire the nonselective trait. In the experiment described in Table 1, hundreds of FA5100 transformants were isolated that had taken up DNA fragments from pL81. In the absence of this

type of analysis, much time was required to isolate transformants of FA5100 that were transformed with the DNA fragment, because the identification procedure was a screening procedure rather than a selection procedure and so all the cells had to be analyzed to determine which were transformants. By having many transformants to analyze, it is now possible to determine the effects of the cloned DNA on lipooligosaccharide production.

This type of experiment can be applied to introduce a variety of nonselective mutations into the gonococcal chromosome. For example, a variety of surface proteins have been cloned from various strains of *N. gonorrhoeae*. However, the mechanism by which their antigenic variation is controlled is unknown. By making specific mutations in the cloned gene before introducing it into *N. gonorrhoeae*, one can determine the role that specific DNA sequences play in regulating the antigenic variation. The usefulness of the congression experiment will be limited by the ability to clone the desired genes and the amount of mismatch, insertion, or deletion the gonococcal recombination system will tolerate.

CONCLUSIONS

The current data indicate that introduction of cloned genes into *N. gonorrhoeae* is not as simple as was initially thought. Owing to the presence of efficient restriction systems, plasmids may be introduced only into strains that lack the enzyme or that do not possess the recognition sequence in their DNA. These problems can be overcome by a judicious choice of strains or through in vitro DNA methylation. Most of the DNA methylases are not commercially available, and their purification is difficult. In addition, no true restriction-negative strains have been described. The transformation-congression experiment has great promise for the introduction of specific mutations, but more work must be done to determine the limits of the system. The final limitation will be in our ability to clone the desired genes.

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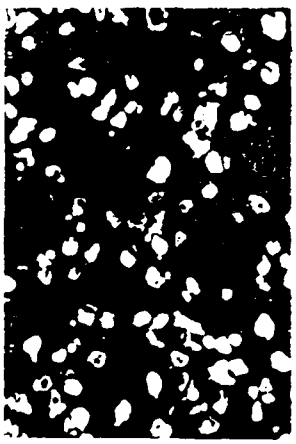

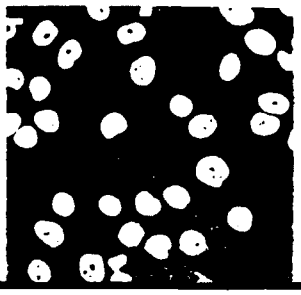

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